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EFFECT OF PRE-TREATMENT OF CHEESE MILK ON THE COMPOSITION AND CHARACTERISTICS OF WHEY AND WHEY PRODUCTS

Doctoral Dissertation

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Faculty of Chemistry and Materials Sciences
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Abstract

Cheese producers want to increase cheese yield. The yield is improved by enhanced transfer of milk proteins and fat to cheese. This requires modifications to the traditional cheese process. During high-temperature heat treatment (HH), whey proteins are partially denaturated and co-precipitated with the cheese matrix. Elevation of the protein concentration of milk enhances the formation of the protein network in which whey proteins and fat are enclosed. The protein concentration is usually increased by ultrafiltration (UF) or microfiltration (MF) membranes. UF concentrates all milk proteins in the cheese milk. With MF, the casein content of milk is increased, as part of the whey proteins are transferred to MF permeate. Often, HH treatment is used in combination with membrane processes. All these milk processing technologies have an effect on the quantity and quality of whey.

Whey is processed into different products, such as demineralised whey powders (DWP), whey protein concentrates (WPC) or isolates (WPI). All of these are used in infant formulas as well as in numerous other applications in food industry. The changes in the cheese process may have an undesirable effect on the composition and functional characteristics of the whey product. In addition to stable functional properties, the nutritional content of the raw material of infant formulas and baby foods is expected to remain within the specification.

In this work, the effects of HH, UF, combination of HH and UF (UFHH) and MF processes on the yield and nutritional quality of whey were studied. The focus was on proteins and amino acids, which are especially important to the infant formula industry. There were no significant differences in the protein and amino acid compositions of the whey products obtained by HH, UF and UFHH. The functional properties of DWP were not adversely affected by these treatments. However, the whey obtained by ceramic and polymeric MF was considered to be of limited use in infant formulas due to its inferior amino acid profile compared to the traditional whey. The applicability of MF cheese whey in other food industries is also questionable, as the gelling properties of whey proteins were adversely affected by the elevated concentration of caseinomacropeptides.

Keywords whey, heat treatment, ultrafiltration, microfiltration, functionality	
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Tiivistelmä

Muutokset juuston valmistusprosesseissa tähtäävät pääasiassa maidon proteiinien ja rasvan saannon sekä juuston tasalaatuisuuden parantamiseen. Kattilamaidon osittaisella korkeakuumennuksella (HH) saadaan lisättyä heraproteiinien denaturoitumista ja saostumista juustomassan sekaan. Kattilamaidon proteiinitason nosto puolestaan tasaa vuodenaikaisvaihtelua sekä nopeuttaa ja tehostaa juoksettumista. Proteiinitasoa nostetaan yleisesti ultrasuodattamalla (UF), jolloin kaikki maidon proteiinit konsentroituvat kattilamaitoon. Mikrosuodatuksella (MF) voidaan nostaa kattilamaidon kaseiinipitoisuutta, koska osa heraproteiineista poistuu MF-permeaattiin. Usein käytetään membraanitekniikoiden ja korkeakuumennuksen yhdistelmiä. Kaikki maidon esikäsittelyprosessit vaikuttavat heran määrään ja laatuun.

Hera prosessoidaan erilaisiksi heratuotteiksi, kuten demineralisoiduksi herajauheeksi (DWP), heraproteiinikonsentraatiksi (WPC) tai –isolaatiksi (WPI). Näitä tuotteita käytetään äidinmaidonkorvikkeiden, lastenruokien sekä erilaisten elintarvikesovellusten raaka-aineina. Kattilamaidon esikäsittelyn aiheuttamat muutokset saattavat aiheuttaa ongelmia muuttuneiden funktionaalisten ominaisuuksien vuoksi. Äidinmaidonkorvike- ja lastenruokateollisuus odottaa raaka-aineiltaan tasaista laatua koostumuksen, ravintosisällön sekä funktionaalisuuden osalta.

Kattilamaidon esikäsittelyiden vaikutusta maidon komponenttien saantoon sekä heran ravintosisältöön tutkittiin määrittämällä kattilamaitojen, herojen sekä jauheiden koostumus. Painopiste oli proteiineissa ja aminohappokoostumuksessa, joka on äidinmaidonkorviketeollisuudelle tärkein proteiinin laadun mittari. HH- UF- ja UFHH- tekniikoilla tuotetut heratuotteet eivät merkittävästi poikenneet verrokkituotteista koostumukseltaan. Näillä tekniikoilla tuotetut DWP-jauheet olivat myös funktionaalisilta ominaisuuksiltaan hyväksyttäviä. Sekä keraaminen että polymeerinen MF tuottivat juustoheraa, jonka proteiinipitoisuus ja –koostumus oli selvästi muuttunut huonommin soveltuvaksi äidinmaidonkorvikkeeseen, johtuen kaseinomakropeptidin (CMP) suuresta osuudesta. Suuri CMP:n osuus heikensi selvästi heraproteiinien geeliytymisominaisuuksia, mikä voi huonontaa MF-heran soveltuvuutta muihinkin elintarvikesovellukseen.

Asiasanat hera, WPC, korkeapastörointi, ultrasuodatus, mikrosuodatus, funktionaalisuus

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Preface

The experimental work for publications I-VII was carried out during 2007-2008. Laboratory scale experiments were conducted at Valio Research and Development, Helsinki, pilot scale tests at the Pilot Dairy of University of Helsinki and Special products factory at Lapinlahti. Process scale experiments were carried out at Valio Ltd, Lapinlahti factory. The experiments in publication VIII were conducted already during 1997-2000 at Valio R&D and Biotechnology and Food Research, MTT Agrifood Research Finland, Jokioinen.

I am grateful to Professor Matti Leisola for waking me up by asking me in 2007 if I had considered doing something useful to finish my studies. I want to thank the Academy of Finland for the funding, and Professor Tiina Mattila-Sandholm, Executive Vice President of Valio R&D for permission and resources to accomplish this. Many thanks to Vice President of Valio R&D, Matti Harju, PhD, and Research Manager Olli Tossavainen, PhD, for encouragement and valuable suggestions. I am deeply indebted to all people in the R&D, production and sales of the Ingredients group, especially PD Manager Pirjo Merimaa, for their support. I thank the reviewers, Professor Ulrich Kulozik, Technical University of Münich and Professor Tapani Alatossava, University of Helsinki, for their comments.

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Finally, I want to thank the most important people in my life, my parents and brothers, and my family Sari, Miia, Mikko and Sanna, for their unconditional support. This is for you Pete, somewhere over the rainbow.

Espoo, December 2009

Marko Outinen

Contents

List of Publications

This thesis consists of an overview and references to the following publications indicated in the text by Roman numerals.

I Outinen, M., Heino, A., Uusi-Rauva, J.O. Pre-treatment methods of Edam cheese milk. Effect on the whey composition, LWT-Food Sci. Techn., *in press,* doi:10.1016/j.lwt.2009.12.001

II Outinen, M., Rantamäki, P., Heino, A.T., The effect of milk pre-treatment on the whey composition and whey powder functionality, J. Food Sci., *in press,* doi:10.1111/j.1750-3841.2009.01382.x.

III Heino, A.T., Uusi-Rauva, J.O., Outinen, M., Microfiltration of milk I. Cheese milk modification by micro- and ultrafiltration and the effect on Emmental cheese quality, Milchwissenschaft 63 (2008) 279-282.

IV Outinen, M., Heino, A.T., Uusi-Rauva, J.O., Microfiltration of milk II. Influence of the concentration factor on the composition of emmental cheese milk and the κ-casein macropeptide content of the whey, Milchwissenschaft 63 (2008) 305-308.

V Heino, A.T, Outinen, M., Uusi-Rauva, J.O., Removal of whey proteins from skimmed milk with polymeric microfiltration membranes, Milchwissenschaft, *in press.*

VI Outinen, M., Heino, A.T, Uusi-Rauva, J.O., Polymeric microfiltration of skimmed milk in Edam cheese process I. Effect of the concentration factor on the composition of vat milk and whey, Milchwissenschaft 65 (2010) 6-11.

VII Outinen, M., Heino, A.T, Uusi-Rauva, J.O., Polymeric microfiltration of skimmed milk in Edam cheese process II. Evaluation of the composition and nutritional quality of whey protein concentrate, Milchwissenschaft, *in press*.

VIII Outinen, M., Rantamäki, P., Whey protein isolate (WPI) gel hardness and structure influenced by the concentration of α-lactalbumin and κ-casein macropeptide Milchwissenschaft 63 (2008) 77-80.

Other relevant publications

Heino, A.T., Uusi-.Rauva, J.O., Outinen, M., Pre-treatment methods of Edam cheese milk. Effect on the cheese yield and composition. LWT-Food Sci. and Techn., *in press*, doi:10.1016/j.lwt.2009.11.004.

Author's contribution

I Marko Outinen planned the experiments, processed the whey and wrote the article.

II Marko Outinen conducted the experiments, processed the whey, and wrote the article.

III Marko Outinen participated in the planning of the experiments and participated in the interpretation of the results and writing of the article.

IV Marko Outinen participated in the planning and executing of the experiments and participated in the interpretation of the results and writing of the article.

V Marko Outinen participated in the planning of the experiments and participated in the interpretation of the results and writing of the article.

VI Marko Outinen participated in the planning of the experiments, processing of whey samples, interpretation of the results and writing of the article.

VII Marko Outinen participated in the planning of the experiments, preparated the WPC samples, interpreted the results and wrote the article.

VIII Marko Outinen produced the whey protein fractions, planned the tests, interpreted the results and wrote the article.

List of Abbreviations

α-LA	α-lactalbumin
Ala	Alanine
Asp	Asparagine
Arg	Arginine
β-LG	β-lactoglobulin
BSA	Bovine serum albumin
CF	Concentration factor
CMP	κ-casein macropeptide, caseinomacropeptide
CN	Casein nitrogen
Cys	Cysteine
Da	Dalton (molecular mass)
DD	Degree of denaturation
DF	Diafiltration
DWP	Demineralised whey powder
EC	Emulsifying capacity (g oil/mg protein)
ED	Electrodialysis
ES	Emulsion stability (%)
FTIR	Fourier Transformation Infrared Spectroscopy
FPLC	Fast protein liquid chromatography
Glu	Glutamic acid
Gly	Glycine
GMP	Glycosylated caseinomacropeptide
HCT	Heat coagulation time (min)
HH	High temperature heat treatment
HH DWP	DWP produced using whey from HH process
HH WPC	WPC produced using whey from HH process
His	Histidine
IGF-I	Human growth factor
IgA,G,M	Immunoglobulin A, G, M
Ile	Isoleucine
IX	Ion exchange
Leu	L-leucine
LF	Lactoferrin
LP	Lactoperoxidase
Lys	L-Lysine
Met	Methionine
MF	Microfiltration
MF WPC	WPC produced using whey from MF process
MH	Medium heat
MW	Molecular weight
N	Nitrogen
NF	Nanofiltration
NPN	Non protein nitrogen

NPN-P	Protein equivalent of non protein nitrogen (N×6.38)
NWP	Native whey protein
NWPC	Native WPC obtained by UF of MF permeate
PKU	Phenylketonuria
pI	Isoelectric point
PP3, 5, 8	Proteose-peptone fractions 3, 5, 8
Pro	Proline
PVDF	Polyvinylidene fluoride
REF DWP	DWP produced using whey from REF process
REF WPC	WPC produced using whey from REF process
RCT	Rennet coagulation time (s)
RO	Reverse osmosis
RP-HPLC	Reverse phase high pressure liquid chromatography
R-SH	Sulfhydryl (thiol) group
RTF	Ready-to-feed
RY	Recovery yield of milk component in permeates or cheese whey (%)
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	Serine
SMP	Skimmed milk powder
T	Temperature (°C)
t	Time (s)
TCA	Trichloric acetic acid
TGF-g2	Human growth factor
Thr	Threonine
TN	Total nitrogen (g/100 g)
TP	Total protein (g/100 g)
Trp	Tryptophan
TS	Total solids (g/100 g)
Tyr	Tyrosine
UF	Ultrafiltration
UF DWP	DWP produced using whey from UF process
UF WPC	WPC produced using whey from UF process
UFHH DWP	DWP produced using whey from UFHH process
UHT	Ultra high-temperature heat treatment
Val	Valine
WHC	Water holding capacity
WP	Whey protein
WPC	Whey protein concentrate
WPI	Whey protein isolate
WPN	Whey protein nitrogen

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Figure 19. Gel hardness of the caseinomacropeptide (CMP) enriched whey protein isolate (WPI) gels in 218 mM Na^+ solution as a function of Ca^{2+} concentration.

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1. Introduction

1.1 Whey production and main components of whey

Sweet whey is a by-product of enzymatic cheese manufacturing process. Cheese milk is subjected to enzymatic treatment with rennin, or chymosin by other name (EC 3.4.23.4.), which attacks the peptide bond between phenylalanine (Phe^{106}) and methionine (Met^{105}) of micellar surface protein κ-casein (Delfour et al., 1965). This protein is embedded in a casein micelle so that the hydrophobic part is inside the micelle, and the hydrophilic part protrudes from the casein micelle. It is important to the stability of the milk protein system, as it keeps the micelles in aqueous phase and apart from each other. As chymosin cuts the specific peptide bond, the hydrophobic part, called κ-casein macropeptide, remains in the solution (whey), as the now hydrophobic caseins unite and form a water-insoluble curd, of which cheese is produced (Kessler, 2002). All milk proteins and other solutes that remain in the aqueous phase after removal of the curd are whey components.

From 10 litres of milk, approximately 1 kg of cheese and 9 kg of sweet whey are obtained. Whey is a very dilute solution: the total solids concentration is 5 to 6 g/100 g of whey, depending on the cheese type and the amount of water added during production of cheese. The total solids of sweet whey consist of lactose (75-77%), protein (12 to 14%), minerals (8 to 9%) and organic acids (2-3%) (Morr and Ha, 1993). Whey protein is a diverse mixture of true proteins, peptides and non-protein (NPN) components. Whey proteins are defined as the components that are soluble at pH 4.6 in their native form (Fox, 2003).

The most abundant whey protein is β-lactoglobulin (β-LG), which consists of approximately 50% of whey proteins (2 to 4 g/kg of whey) and 12% of the total proteins in milk (Figure 1). The most common genetic variants of β-LG are A and B, other variants C, D, E, F and G has been found (Fox, 2003). The molecular weight (MW) of β -LG is ca.18.3 kDa consisting of 162 amino acids (Fox, 1989). It is a dimer at pH 5.2-7.5 (36 kDa), octamer (147 kDa) at pH 3.5-5.2 and monomer (18 kDa) at pH less than 3.5. The isoelectric point (pI) of β-LG is 5.35-5.49. Human milk does not contain β-LG (Eigel et al., 1984). β-LG is largely responsible for the functional properties of whey proteins. It contains five cysteine (Cys) residues, of which four are linked with each

other with covalent disulphide (S-S) bonds, leaving one highly reactive free sulfhydryl (thiol) group (R-SH).

The concentration of α-lactalbumin (α-LA) in bovine milk is 1 to 1.5 g/kg, consisting 15 to 20% of whey proteins and 4-5% of the total proteins in milk (Figure 1). The MW of α-LA is ca. 14 kDa, the pI 4.8. Of the genetic variants A, B ja C of α-LA, only variant B exists in bovine milk. Variants A and B consist of 123 and variant C of 124 amino acids (Fox, 2003). α-LA is a metalloprotein and capable of binding Al^{3+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Na^{+} ja K^{+} -ions. Ca^{2+} is an essential structural component of native α-LA (Brew ja Grobler, 1992). α-LA is a major protein component in human milk (Eigel et al., 1984). There is no free thiol group in α-LA, since it contains four Cys residues, all of which all are involved in disulphide bonding.

κ-casein macropeptide (caseinomacropeptide or CMP) is the largest group of glycosylated proteins in whey, and their application possibilities have been studied extensively (Kreuss and Kulozik, 2006). The amount of κ-casein of total casein is 12-15% (Figure 1), and of this approximately 40% is enzymatically cleaved into CMP (Swaisgood, 2003a; Swaisgood, 2003b), resulting in an average CMP yield of 50-58 g/kg casein, consisting of 20-25% (1.2-1.5 g/kg) of total proteins in rennet whey (Marshall, 1991; Thomä-Worringer et al., 2006). The content of the glycosylated CMP (GMP) ranges from 30% (Casal et al., 2005) to 60% (Lieske and Konrad, 1996; Vreeman et al., 1986) of total CMP. The pI of the GMP molecule is in a range of 2.8-4.1, whereas the pI of the non-glycosylated CMP is 4.2-4.3. At the pI, the CMP molecule loses the net charge and consequently the layer of water molecules, resulting in reduction of the apparent MW from 20 to 8 kDa (Kawasaki et al., 1993, Lieske et al., 2004a). Caseinomacropeptides are not affected by high-temperature heat treatment at the pH of milk, but under acidic conditions it is deglycosylated (Lieske et al., 2004b; Lieske et al., 2004c). Total caseinomacropeptides are referred in this text as CMP, and the glycosylated part as GMP.

The concentration of bovine serum albumin (BSA) is 0.1-0.4 g/kg, consisting of 3-10% of bovine whey proteins (de Wit, 1998; Fox, 2003). The concentration is similar in human milk (Davies, 1974). The MW of BSA is 66 kDa and pI 5.1. BSA is a large protein consisting of 582 amino acids. The human BSA consists of 585 amino acids (Fox, 2003). In addition to 17 Cys residues, BSA has one free thiol group (-SH),

enabling the BSA molecule to self-aggregate, like β-LG, or form covalent bonds with β-LG and other proteins (Morr and Ha, 1993).

The immunoglobulins are a diverse group of proteins. There are four main types of immunoglobulins in bovine milk, IgA, IgM and IgG_1 and IgG_2, of which ca. 85% is IgG_1 (Lindström et al., 1994). The concentration of immunoglobulins in bovine milk is 0.6-1.0 g/kg (Mehra et al., 2006; de Wit, 1998). It consists of four subunits, two light chains and two heavy chains. The MW of IgG is 150 kDa (Fox, 2003). Even though bovine IgG is very heat labile (Figure 3) it remains intact during normal pasteurisation of 72°C for 15 s according to Mainer et al. (1997).

The concentration of lactoferrin (LF) in bovine milk is 0.02-0.2 g/kg (Steijins and Hooijdonk, 2000; de Wit, 1998). Bovine LF is a large protein molecule (MW 80 000 Da), with 689 amino acid residues (34 Cys residues) (Pierce et al., 1991). LF consists of four domains, N1, N2, C1 and C2, between which ferric iron (Fe^{3+}) is bound (Steijins and Hooijdonk, 2000). The theoretical pI value of LF is 9.4 (Steijins and Hooijdonk, 2000).

According to Treblay et al. (2003), the non-protein nitrogen fraction (NPN) is defined as the nitrogenous components that are soluble in 12% trichloric acetic acid (TCA). Some NPN components are indigenous to milk, some are formed during the cheese process (Figure 1). Proteose-peptones (PP), which are formed from β-casein by plasmin action, and κ-casein macropeptides, or caseinomacropeptides, are only partially (approximately 70%) soluble in 12% TCA (van Bockel, 1993) and accordingly belong partially to protein and partially to the NPN fraction.

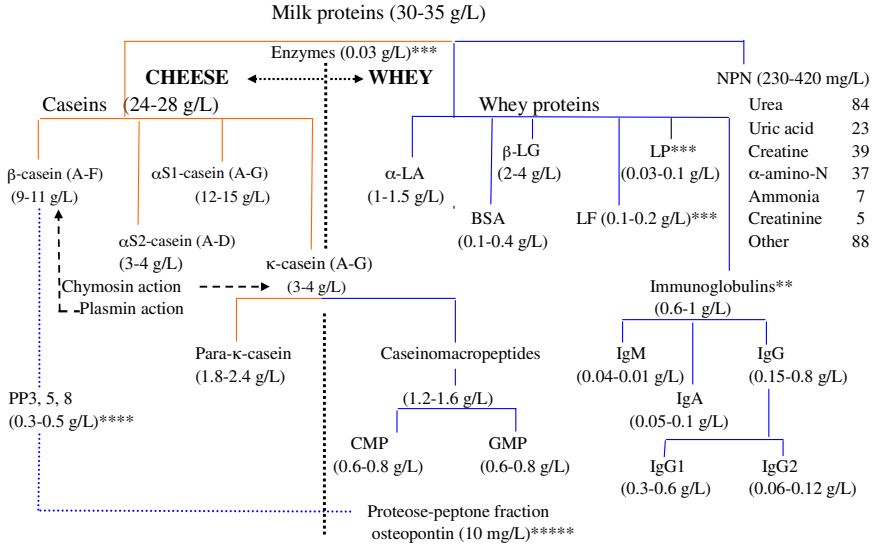


Figure 1. The concentration of main milk protein components and their division into cheese and whey fractions. The data was adapted from Swaisgood (2003), *Fox (2003), ** Mehra et al. (2006), ***de Wit (1998) and ****Treblay et al. (2003), *****Sorensen and Pedersen (1993). CMP=non-glycosylated caseinomacropeptides, GMP = glycosylated caseinomacropeptides. LF=lactoferrin, LP=lactoperoxidase, PP=proteose-peptone fraction α-LA=α-lactalbumin, β-LG=β-lactoglobulin, BSA=bovine serum albumin.

1.2 Whey processing and whey products

The whey must be clarified from casein and fat residues, concentrated, demineralised and dried in order to be usable in the food industry (Pearce, 1992; Hoppe and Higgins, 1992). The end product may be demineralised whey powder (DWP), protein-enriched whey protein concentrate or isolate (WPC/WPI), or protein fractions (Figure 2). This work does not discuss lactose or lactose derivates, as they have been exhaustively reported by Harju (1991).

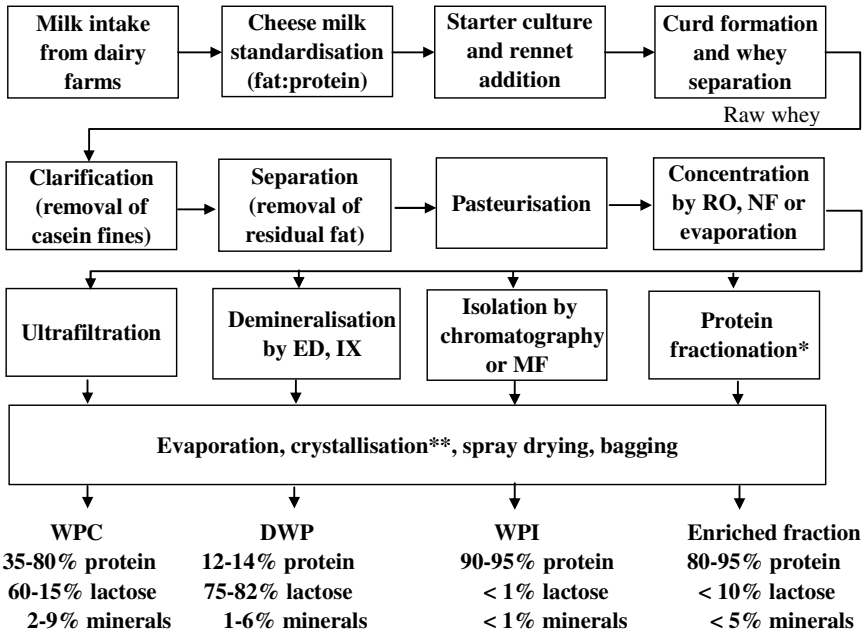


Figure 2. Whey processing and the composition of major whey products. ED = electrodialysis, RO = reverse osmosis, NF = nanofiltration, IX = ion exchange, MF = microfiltration. * = Commercially available reasonably pure fractions of α-LA, β-LG, CMP, lactoferrin (LF), lactoperoxidase (LP) are available. ** = Crystallisation is necessary only for DWP because of high lactose content.

1.3 Use of whey products as ingredients in infant formula

Whey products are an especially important ingredient for baby food and infant nutrition industry due to nutritionally valuable whey proteins (de Wit, 1998). Typical infant formula consists largely (50-60%) of whey solids (Mulchandani et al., 1979) and its composition is strictly outlined by EU legislation (Anon., 2006) based on recommendations by nutrition scientists (Koletzko et al., 2005). The amino acid composition of WPC or DWP is important, since the amount of essential amino acids per unit of energy of infant formula are specified in the directive (Table 1). The quantity or quality of proteins is not strictly defined. Therefore, from the nutritional point of view, the effect of pre-treatments of milk would be significant for whey producer only if it would affect the amino acid composition. Each essential amino acid plays a specific

role in the human metabolism. For example, tryptophan (Trp) acts as a precursor of sleep-inducing neurotransmitter serotonin (Thomä-Worringer et al., 2006), and cysteine (Cys) is a component of glutathione, which prevents oxidative damage (de Wit, 1998).

Table 1. Amino acid compositions (mol-%) of α-LA, β-LG, BSA (Eigel et al., 1984), CMP (Tanimoto et al., 1992), IgG_1 (Larsson, 1992) and LF (Pierce et al., 1991).

	α-LA	β-LG	BSA	CMP	IgG1	LF
Asparagine (Asp)	17.07	9.26	8.76	7.81	7.94	9.43
Threonine (Thr)**	6.50	4.94	5.84	18.75	9.40	5.22
Serine (Ser)	5.69	4.32	4.81	9.38	14.17	6.53
Glutamic acid (Glu)	10.57	15.40	13.40	15.63	8.69	10.01
Proline (Pro)	1.63	4.94	4.81	12.50	7.13	4.35
Glycine (Gly)	4.88	2.47	2.75	1.56	6.94	6.97
Alanine (Ala)	2.44	9.26	7.90	7.81	4.97	9.72
Valine (Val)**	4.88	5.56	6.19	9.38	9.42	6.82
Cysteine (Cys)***	6.50	3.09	6.01	0.00	3.40	4.93
Methionine (Met)**	0.81	2.47	0.69	1.56	0.91	0.58
Isoleucine (Ile)**	6.50	6.17	2.41	9.38	2.51	2.18
Leucine (Leu)**	10.57	13.58	10.48	1.56	6.28	9.43
Tyrosine (Tyr)***	3.25	2.47	3.26	0.00	3.60	3.19
Phenylalanine (Phe)**	3.25	2.47	4.64	0.00	2.59	3.92
Tryptophan (Trp)**	3.25	1.23	1.23	0.00	1.79	1.89
Lysine (Lys)**	9.76	9.26	10.14	4.69	5.58	7.84
Histidine (His)***	2.44	1.23	2.92	0.00	1.70	1.31
Arginine (Arg)***	0.81	1.85	3.95	0.00	3.93	5.66

*)Minimum content in infant formulae; **Essential amino acid for all humans;
***Essential for infants and growing chidren (Young, 1994).

1.4 Importance of functional properties of whey products

Equally important for the ingredient producer is to make sure that changes in cheese manufacturing processes do not affect processing characteristics or the physicochemical quality of whey products. Protein solubility and heat stability are very important especially for ingredients used in Ultra High Temperature (UHT) treated products such as ready-to-feed (RTF) formulas (McSweeney et al., 2004). Deviations in the emulsifying capacity and emulsion stability of DWP may result in fat separation during storage (creaming) of UHT products (Moon and Mangino, 2004). Bottle feeding sets certain limits on the viscosity of RTF products (Moon and Mangino, 2004). Functionality of whey powders is affected also by non-protein chemical components. Fat affects the emulsifying properties (Vaghela and Kilara, 1996), whereas elevated Na^+ and Ca^{2+} concentrations have been reported to impair solubility and heat stability of

whey proteins (Hidalgo and Gamper, 1977). De Wit (1981) reported that precipitation of whey proteins is caused when concentration of divalent cations Ca^{2+} and Mg^{2+} is sufficient to neutralise the negative net charge of the proteins, inducing isoelectric precipitation. Isoelectric precipitation occurs at the pI of the protein via electrostatic bonding. The control of ionic environment and pH is therefore crucial for the stability of whey protein solutions (such as DWP). If the net charge of the protein (i.e. the difference between pI of the protein and the pH of the solution) is high, the proteins tend to form linear aggregates with high electrostatic repulsion between the charged molecules. These molecules are less likely to precipitate, especially in low concentration of divalent cations (Singh and Havea, 2003; Morr and Ha, 1993). Impaired solubility and heat stability in turn impair the emulsification characteristics (Morr 1979; Yamauchi et al., 1980).

The effect of concentration and the interactions of whey proteins, mainly β-LG with α-LA or BSA on gelling properties has been studied extensively (Hillier et al., 1980; Hines and Foegeding, 1993; Kornhorst and Mangino, 1985; Matsudomi et al., 1992; Matsudomi et al., 1993; Matsudomi et al., 1994; Foegeding et al., 1995; Gezimati et al., 1996; Gezimati et al., 1997; Calvo et al., 1993). Especially the presence of CMP has been reported to have a deteriorating effect on the gel hardness of whey protein isolates (WPI) and concentrates (WPC) (Kornhorst and Mangino, 1985; Langley and Green, 1989; Veith and Reynolds, 2004). Contradictory, CMP has also been found to improve the properties of whey protein gels (Gault and Fauquant, 1992; Martin-Diana et al., 2004; Doi, 1993). Ionic conditions, pH and thermal history have a significant impact on gelation characteristics (de Wit, 1989; de Wit et al., 1986; de Wit et al., 1988; Gault and Fauquant, 1992; Margoshes, 1990; Matsudomi et al., 1991). Maximum whey protein gel hardness at 20, 100-300 and 200 mM Na^{+} concentrations were reported by Matsudomi et al. (1991), Schmidt et al. (1978) and Mulvihill and Kinsella (1988). Maximum hardness was achieved at 11.1 and 20 mM Ca^{2+} concentrations by Schmidt et al. (1979) and Zirbel and Kinsella (1988), respectively. pH has a significant effect on the appearance and structure of the gels. From pH 4 to 7, particulate gels, whereas from pH 7 to 9 fine-stranded gels are formed (Aquilera, 1995). Very small deviations in neutral pH may lead to significant structural changes (Doi, 1993).

1.5 High-temperature heat treatment (HH) of cheese milk

Several approaches to increase cheese yield by pre-treating the cheese vat milk have been studied and some of them are already in commercial use. Industrially viable cheese vat milk pre-treatment methods include high-temperature heat treatment (HH), ultrafiltration (UF) and microfiltration (MF).

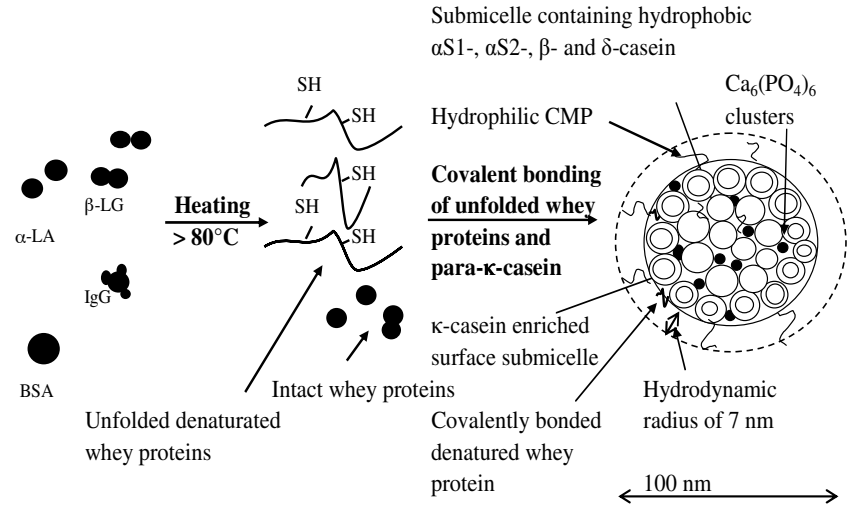
At traditional pasteurisation conditions of 72-75°C for 15 s., non-covalent reactions between whey proteins, mainly β-lactoglobulin (β-LG) and micellar surface protein κ-casein occur (Jang and Swaisgood, 1990). After pasteurisation approximately 5% of whey proteins are associated with casein (Lau et al., 1990), and the effect on the renneting, or rennet coagulation time (RCT) is limited (Lucey, 1995). However, when milk is heated at temperatures above 80°C, the tertiary structure of whey proteins begins to unfold revealing intramolecular highly reactive thiol groups, broken disulphide and hydrophobic bonds (Figure 3a), which form covalent and hydrophobic bonds with themselves or with caseins, especially κ-casein (Zittle et al., 1962; Sawyer et al., 1963; Sawyer, 1969; Dalgleish, 1990), when associated with caseins in milk. This is possible for whey proteins with sulphur containing amino acids with thiol groups (R-SH) which can form covalent bonds, such as β-LG and BSA (one free SH group). As α-LA does not contain free thiol groups, it does not associate with caseins (Eigel et al., 1984; Morr and Ha, 1993). However, α-LA is associated with caseins in the presence of β-LG during heating, via formation of α-LA-β-LG complexes (Morr and Ha, 1993). In addition to covalent bonding, the denatured, unfolded whey proteins may attach to the casein micelle surface after renneting, by means of hydrophobic interaction, when the surface of the micelle has changed from hydrophilic to hydrophobic (Figure 3b).

Formation of β-LG-κ-casein complex affects the cheese manufacturing process negatively in two ways. First, because of physical blockage of the target peptide bond, the chymosin action is impaired resulting in increased RCT (van Hooydonk, 1987; Lucey, 1993). Secondly, since the curd formation is based on the removal of the hydrophilic part of κ-casein and subsequent repulsion of the aqueus layer (Kessler, 2002), the hydrophilic whey proteins complexes impair this reaction. Samuelsson et al. (1997b) found that the heat treatment of microfiltered retentate also resulted in reduced rate of curd formation and gel strength, but did not affect the formation of CMP.

The β-LG-κ-casein complex is mainly formed between sulphur containing cysteine (Cys) residues (Sawyer, 1963). As Cys is present only in the para-κ-casein section of κ-casein, the complex remains in the curd after renneting (Kessler, 2002). Hence, the possibility of increasing the cheese yield by incorporating whey proteins into casein matrix has been intensively studied. It has been found that the negative effects of HH treatment - i.e. retardation of renneting and reduction in gel strength - may be compensated by altering the processing conditions, such as pH or addition of calcium (Lucey et al., 1993).

HH is widely utilised in the production of fresh and semi-soft cheeses (Banks et al., 1993; Singh and Waungana, 2001). Aggregated denaturated whey proteins are transferred to cheese matrix (Pedersen and Ottosen, 1992; McMahon et al., 1993), resulting in improved cheese yield and changes in the whey protein composition (Hinrichs, 2001). Some proteins may also remain in whey as denatured, soluble aggregates (Dalgleish, 1990), which may affect the functionality of the whey product.

a. Heat treatment of milk at natural pH



b. Renneting

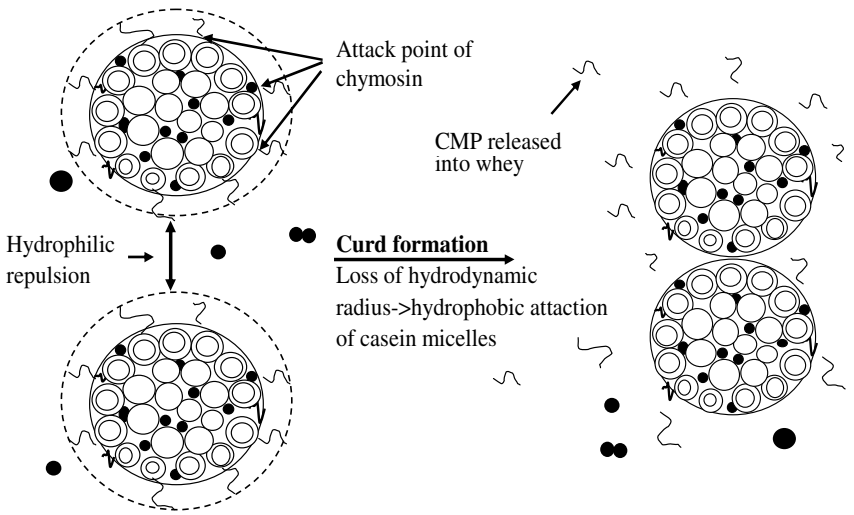


Figure 3. The effect of heat treatment on the molecular structure of β-LG, formation of covalent bond with κ-casein (a) and the effect of renneting on the micellar casein (curd formation) (b). Data adapted from Zittle (1962), Sawyer et al. (1963), Walstra (1990), de Wit (1998), Fox (2003) and Kessler (2002).

β-LG is denatured irreversibly and is more easily aggregated than α-LA, which is capable of almost complete renaturation upon cooling (Dannenberg and Kessler, 1988a; Dannenberg and Kessler, 1988b; Rüegg et al., 1977). Minor protein components bovine serum albumin (BSA), lactoferrin (LF) and immunoglobulin G (IgG) are even more readily denatured (Law et al., 1994), as shown in Figure 4. According to Dannenberg and Kessler (1988a), the high-temperature heat treatment of skimmed milk at 93°C for 15 s, as was used in this work, would denature approx. 50% of β-LG, but only approximately 5% of α-LA of the heat-treated portion of milk, and therefore could have an effect on the subsequent whey protein composition and functionality.

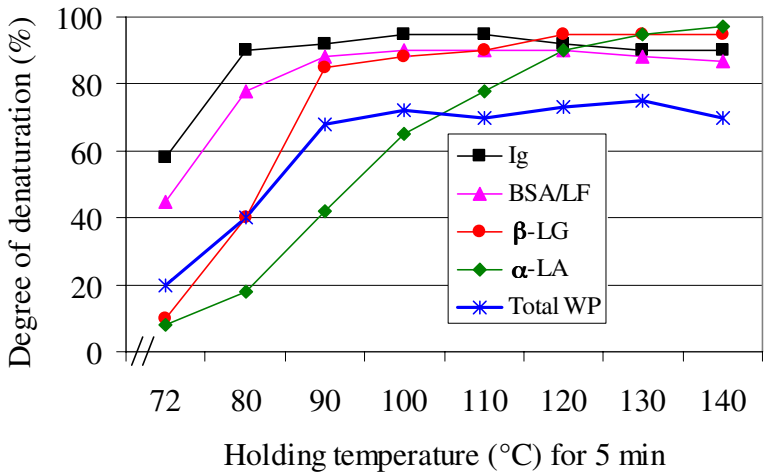


Figure 4. Degree of denaturation (DD) of the individual and total whey proteins (Total WP). Ig=Immunoglobulins, BSA=bovine serum albumin, LF=lactoferrin, β-LG=β-lactoglobulin, α-LA=α-lactalbumin (adapted from Law et al., 1994).

1.6 Ultrafiltration (UF) of cheese milk

Increasing milk protein concentration by ultrafiltration (UF) is one possibility to overcome the retardation of renneting properties of casein micelles, which have been coated with heat denatured whey proteins. The gel strength decreases due to blocking of the enzyme attack point by the whey proteins, but increases when milk is concentrated (Hinrichs, 2001). The increased whey protein in cheese matrix was suggested to be caused by steric inclusion of native whey proteins in the rennet-induced gel network

already by Bachmann et al. (1975), and confirmed by Singh and Waughana (2001). Considerable cheese yield increases have indeed been reported (Maubois and Mocquot, 1975; Brown and Ernstrom, 1982; Guinee et al., 1994; Guinee et al., 2006; Banks et al., 1993). The rate of curd formation (milk coagulation) has been reported to accelerate with increasing protein content of milk (St-Gelais and Haché, 1995; Caron et al., 1997). Guinee et al. (1994) reported that when the protein content of the cheese milk exceeded 50 g/l, the curd formation and cutting was difficult to control, and led to casein and fat losses to whey, whereas fat was more efficiently retained in the cheese matrix at milk protein content of 30-40 g/l. A linear relationship for the protein content of cheese milk and corresponding whey was also found.

All true whey proteins are retained in the UF retentate of milk, whereas the non-protein nitrogen (NPN) is partly lost to the UF permeate, equalling ca. 5% loss of the total nitrogenous substances of milk. The casein to whey protein ratio of the UF retentate remains unaltered (Maubois and Mocquot, 1975), but the increased density of casein matrix enhances co-precipitation of whey proteins with casein, thereby altering the composition of whey (Ardisson-Korat and Rizvi, 2004; Guinee et al., 2006). When concentrated milk has been used for cheese production, increase of protein and fat content in whey has been reported (St-Gelais and Haché, 1995; St-Gelais et al., 1995).

1.7 High-temperature heat treatment with ultrafiltration (UFHH)

The combination of high-temperature heat treatment with ultrafiltration (UFHH) increases the whey protein yield to cheese matrix by two mechanisms. First, the steric inclusion of the native whey proteins into the cheese curd is enhanced due to stronger casein network formation. Secondly, the co-precipitation of the denatured whey proteins, especially β-LG, is enhanced as the protein content of total solids is increased, as part of the non-protein solids such as minerals and lactose are transferred to UF permeate. Lactose has been found to protect especially β-LG against denaturation and aggregation by forming a hydrated layer of lactose around the protein molecule, the α-LA molecule is less protected (Plock et al., 1987; Plock et al., 1988a; Plock et al., 1988b). HH treatment (95°C for 5 min) in combination with UF has been used succesfully in the fresh cheese production, where considerable yield increases have been observed compared to the traditional thermo-separator process (Pedersen and Ottosen, 1993).

1.8 Microfiltration (MF) of cheese milk

When the vat milk is concentrated by microfiltration, the casein to total protein ratio of vat milk is increased, since whey proteins have been partly removed to MF permeate. (Samuelsson et al., 1997b; Maubois, 1997; Neocleous et al., 2002a). The separation of WP and caseins is based on differences in molecular size (Figure 5), though several other factors affect the process (Gésan-Guiziou et al., 2000; Saxena et al., 2009).

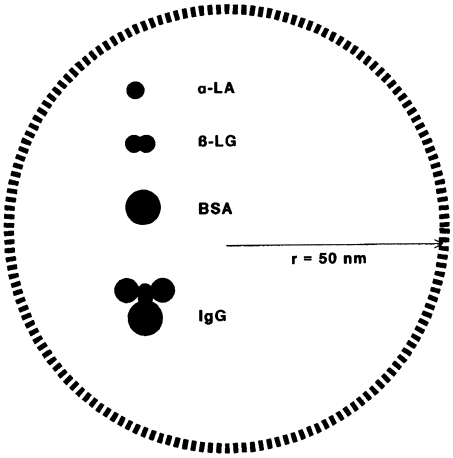


Figure 5. Size comparison of an average casein micelle (100 nm) and whey proteins α-lactalbumin (α-LA) (1.8 nm), dimer of β-lactoglobulin (β-LG) (3.5 nm), bovine serum albumin (BSA) (4 nm) and immunoglobulin G (IgG) (6 nm) (de Wit, 1998).

In addition to the possibility of eliminating the yearly fluctuation in casein concentration (Neocleus et al., 2002a; Neocleus et al., 2002b), the use of casein-enriched MF retentate has been reported to result in a more efficient and faster curd formation (Samuelsson et al., 1997a) and in elevated (2-4%) cheese yield (Maubois et al., 2001; St-Gelais et al., 1995; Papadatos et al., 2003). The volume of cheese whey has been reported to be reduced by 22-26% (Maubois et al., 2001), and the quantity of whey protein in the cheese whey is reduced. In addition, there is a possibility that variations in specific mass fluxes of individual whey proteins through the MF membrane may result in altered whey protein composition. The differences in permeability of proteins was utilised by Akbache et al. (2009) in a fractionating growth factors TGF-g2 from IGF-I. Punidadas and Rizvi (1998) reported that only α-La and β-LG were detected in their skimmed milk MF permeate obtained with 0.05 μm ceramic membranes. Significant differences

in permeability was observed also by Le Berre and Daufin (1996), Tolkach and Kulozik (2005) and Jost et al. (1999). In most studies, however, the permeability of individual proteins has not been reported (Samuelsson et al., 1997b, Marcelo and Rizvi, 2008), and in some reports differences were not detected. (Britten and Pouliot, 1996; Maubois et al., 2001).

As quantitative hydrolysis of κ-casein is required for the proper coagulation of the casein micelles (Walstra, 1990), the formation of CMP from casein is constant regardless of the pre-treatment method. Thus, partial removal of whey proteins by MF before renneting induces a significant increase in the CMP concentration (Maubois, 2002; Kulozik and Kersten, 2002). Maubois et al. (2001) observed a 22-26% decrease in cheese whey volume and 20% increase in the CMP concentration of total proteins.

Production of whey product for infant nutrition from this type of whey may be problematic, since the amino acid compostion of CMP is very different from the other protein components in whey. It lacks the essential amino acids Cys, Phe, Tyr, Trp His and Arg, but is rich in Thr (Tanimoto et al., 1992). People suffering from phenylketonuria (PKU) - an inborn error of the metabolism - must restrict their intake of Phe (Purnell, 2001). As all genetic variants of CMP lack the aromatic amino acids (Swaisgood, 2003c), the CMP-enriched whey has been suggested as raw material for dietary food supplement for PKU (Marshall, 1991; Ney, 2005). Maximum practical Phe content on a dietary point of view for such a product is 2-4 mg/g CMP (Lim et al., 2007), which would require ca. 90% reduction of the Phe content in whey protein.

1.9 The aim of this work

Only a few reports on changes in the whey composition and functionality induced by pre-treatment methods of vat milk has been published (Peri et al., 1985; St-Gelais et al., 1995). The aim of this work was to evaluate the effects of the high temperature heat treatment (HH), ultrafiltration (UF) (study I, II) and microfiltration (MF) (study III-VII) on the mass balance and quality of the whey solids in cheese processes as well as to evaluate the changes in compositional and nutritional value of subsequent whey and whey products. Excessive diafiltration was carried out with polymeric MF membranes (study IV-VII) in order to study the effect of the concentration factor on the whey composition, i.e. how much of the whey proteins could be removed from skimmed milk

prior to cheese production, emphasis being in maximising the relative CMP content in cheese whey.

The effect of HH and UF of cheese milk on the degree of denaturation, heat stability, water binding capacity, emulsion capacity and stability of DWP was studied (study II). In order to study the functionality CMP-enriched whey produced from the MF process, WPI gel hardness with elevated CMP concentrations, in controlled ionic environments, was studied (study VIII).

2 Materials and methods

2.1 Pre-treatment of milk

The raw milk was separated, pasteurised and cooled before further processing as described in studies I-VII.

2.2 High temperature heat treatment (I, II)

Part of the pasteurised, skimmed milk (25%) was subjected to an additional HH treatment (93ºC, 15 s). The HH treated milk was cooled and mixed in the cheese vat with the rest of the pasteurised milk as described in study I and II (Figure 6)

2.3 Ultrafiltration

Pasteurised skimmed milk was concentrated in a pilot (study I) or production scale (study II) ultrafiltration (UF) unit as described Figure 6. Laboratory scale UF unit (studies I, III-VII) was used to concentrate the clarified wheys (Figure 8). In all UF units, 10 kDa membranes were used.

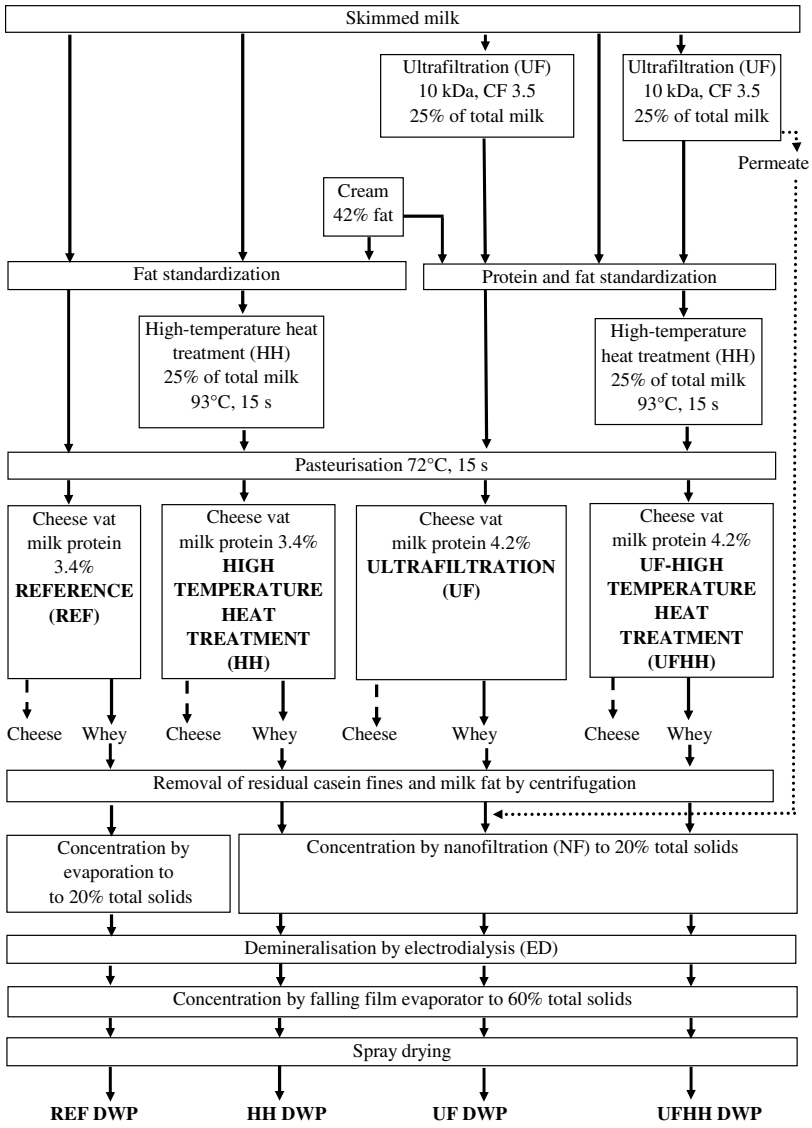


Figure 6. The process flow charts of the pre-treatment of skim milk and recombination of the high temperature heat treated (HH), ultrafiltered (UF) and high-temperature heat treated ultrafiltered (UFHH) cheese vat milks and wheys, and processing of the subsequent whey into demineralised whey powders (DWP).

2.4 Microfiltration

2.4.1 Microfiltration of milk with ceramic membranes (III, IV)

To produce MF retentate for Emmental cheese production, skimmed milk was microfiltered with 0.1 μm ceramic membranes as described in study III.

2.4.2 Microfiltration of milk with polymeric membranes (I, V-VII)

Microfiltration to concentration factor 4

Skimmed milk was microfiltered with spiral wound 800 kDa polymeric polyvinylidene fluoride (PVDF) membranes in a pilot plant in batch filtration mode with a CF of 4. The target protein content of the retentate was 120 g protein/kg retentate. To obtain CF 10 and CF 70 in studies III-VII, the CF 4 retentate was further diafiltered with UF permeate.

Diafiltration by ultrafiltration permeate to obtain concentration factor 70

Diafiltration (DF) was carried out using UF permeate. After microfiltration to CF 4, permeate was added and filtration was continued. UF permeate was obtained by ultrafiltering the MF (CF 4) permeate of skimmed milk in a pilot plant. The total concentration factor was calculated as the CF value multiplied with diafiltration CF value (Equation 1)

$$\mathrm{CF}\,(-) = \left(\frac{\text{feed}\quad(\mathrm{L})}{\text{retentate}\quad(\mathrm{L})}\right) \quad \mathrm{x} \quad \left(\frac{\text{diafiltration feed}\quad(\mathrm{L})}{\text{diafiltration retentate}\quad(\mathrm{L})}\right) \tag{1}$$

2.4.3 Recombination of the HH, UF and UFHH cheese milks (I, II)

The vat milks were standardised for protein and fat as described in I and II (Figure 6).

2.4.4 Recombination of MF milks (III-VII)

Cheese milk was recombined using MF retentate, UF permeate of the MF permeate, water and cream (Figure 7). The aim was to equalise the casein and fat-to-protein ratio in each experiment. The protein concentration of the MF milks was adjusted to 42 g/kg with MF retentates. The protein concentration of the reference milk was 34 g/kg milk.

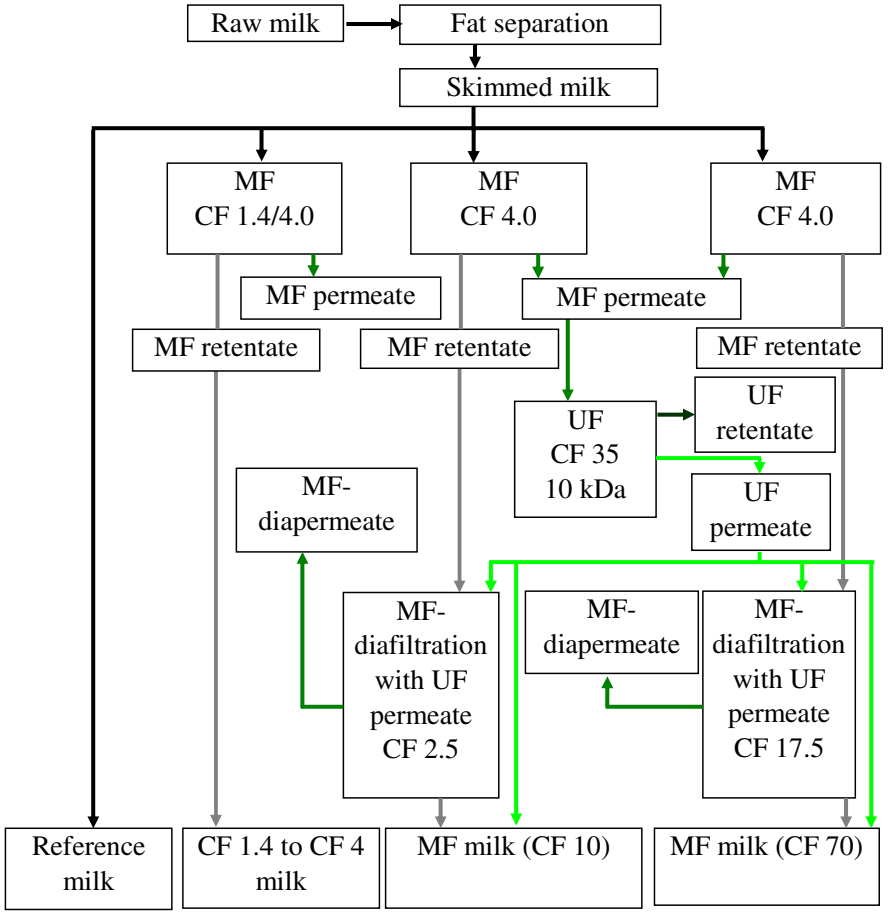


Figure 7. Recombination of the MF milks with retentates obtained by 0.1 µm ceramic (study III-IV) or 800 kDa polymeric (study V-VII) microfiltration (MF). CF=volume concentration factor, UF=ultrafiltration (10 kDa), DF=Diafiltration.

2.5 Production of freeze-dried WPC powders (I, III-VII)

2.5.1 Collection and sampling of whey

After coagulation and separation of the curd, a 20 L sample of whey was collected from each vat, and stored at 5ºC for further processing. Smaller aliquots for compositional analysis were taken and rest of the whey was discarded.

2.5.2 Removal of residual fat and casein

In order to minimise the effect of residual casein fines on the amino acid analysis, whey was clarified with ceramic 0.8 μm MF membranes according to Figure 8. Clarified whey (MF permeate) was collected for further processing.

2.5.3 Concentration and freeze-drying of whey

The WPC was produced by ultrafiltering the clarified whey (0.8μm MF permeate) with spiral wound 10 kDa UF membranes with a CF 20 (Figure 8). The native WPC (NWPC) was produced by ultrafiltering the MF permeate of skimmed milk. Since the MF permeate was dilute compared to whey, CF 35 was used. The UF retentates were freeze-dried.

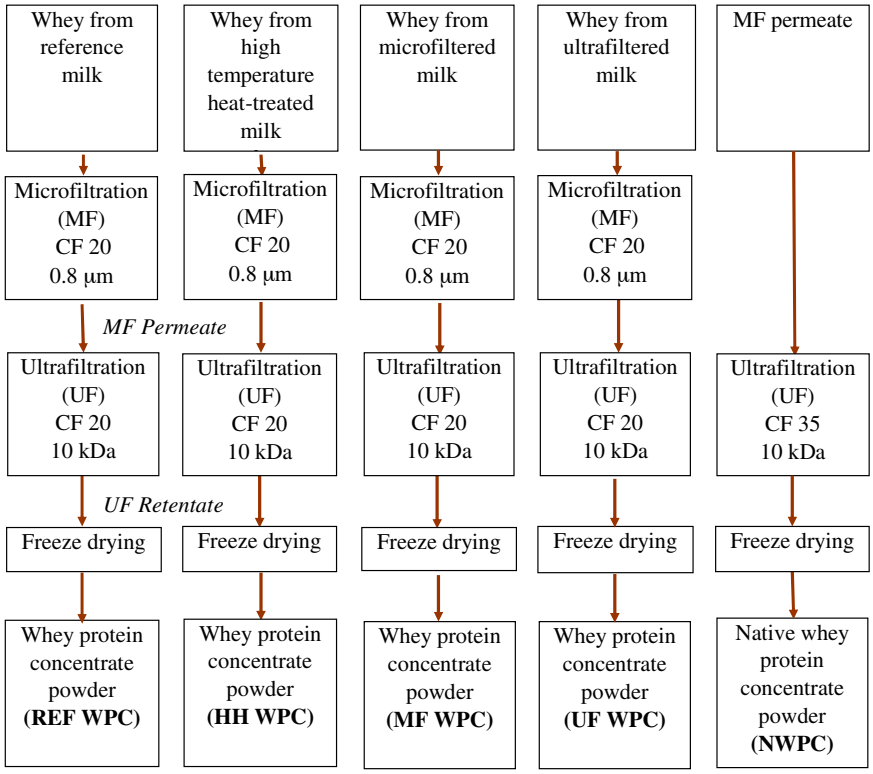


Figure 8. Production of whey protein concentrate (WPC) powders. Whey was obtained from reference (REF WPC), high temperature heat-treated, microfiltered (MF WPC 1.4 to 70) or ultrafiltered milk (Figures 6 and 7). The native WPC (NWPC) was produced from the MF permeate.

2.6 Production of the demineralised whey powders (II)

2.6.1 Collection and sampling of whey

After separation of the curd, whey was clarified, cooled and stored at 5ºC for maximum of 5 h before nanofiltration (NF). Four aliquots were taken from each batch of clarified whey during the production day. The mean of these four aliquots represent the result of one batch. The whey was processed to DWP as described in Figure 6.

2.6.2 Pre-concentration of whey

Clarified whey was nanofiltered in continuous mode or evaporated to reach TS content of 20-21 g/100 g retentate.

2.6.3 Electrodialysis (ED) of the whey concentrate

The whey concentrate was demineralised by electrodialysis (ED), as described by Hoppe and Higgins (1992). ED was terminated after the product solution conductivity of 650 μS was reached.

2.6.4 Drying of the demineralised whey concentrates

The demineralised concentrates were pasteurised at 78°C for 5 min, further evaporated in a four-stage falling film evaporator to a TS content of 60%, and finally crystallised and spray-dried as described in study II. The DWP samples were taken immediately after the final fluid bed. This procedure ensured that the powder sample matched exactly with the corresponding whey sample. Possible structural changes caused the mechanical handling of the powder during transport and storage were also avoided.

2.7 Methods of analysis

2.7.1 Chemical analysis

Total solids (TS) were determined according to IDF 21B:1987. Fat content of milk and cream was analysed using IDF 1C/16C:1987, fat content of whey using IDF 1D:1996. Lactose was determined enzymatically with modified IDF 79-2:2002. Total nitrogen (TN) was determined according to IDF 20-1:2002; total protein (TP) = TN × 6.38. Non-protein nitrogen (NPN) was analysed with Kjeldahl after precipitation with 12% TCA solution (IDF 20-4:2002). NPN was converted to NPN protein equivalent NPN-P by multiplying by a factor of 6.38. Casein nitrogen (CN) was determined according to IDF 29:1964. Whey protein (WP) was calculated using formula WP = (TN – CN – NPN) × 6.38. The conversion factor is dependent on the nitrogen content of the compound, which is dependent on the amino acid composition and the degree of glycosylation (Treblay et al., 2003), and is thus slightly different for each protein, ranging from 5.72 of non-glycosylated lactoferrin to 7.36 (Anon., 2006b; van Bockel, 1993) of the caseinomacropeptide. With purified protein fractions (study VIII), conversion factor of 7.36 (van Bockel, 1993) was used for CMP fraction and 6.38 for β-lactoglobulin (van Bockel, 1993) and WPI fraction (essentially β-lactoglobulin). In studies I-VII, an

average conversion factor of 6.38 was used, which may underestimate the protein content of CMP enriched samples.

In studies I-VII, whey proteins α-LA, β-LG, CMP and GMP were determined with a reverse phase high pressure liquid chromatography (RP-HPLC) method (Thomä et al., 2006). In study VIII, whey proteins were analysed by fast protein liquid chromatography (FPLC) according to Syväoja and Korhonen (1994). The FPLC analysis of CMP required precipitation of proteins with 8% trichloroacetic acid (TCA), and the CMP eluted in single peak. Amino acids were determined at EuroFins (Lidköping, Sweden) according to ISO 13903:2005, excluding Trp, which was determined according to ISO 13904:2005. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed at the VTT Technical Research Center of Finland according to Laemmli (1970). Minerals were determined in studies I-VII using inductively coupled plasma mass spectrophotometer (MS-ICP, Elan 6100, Perkin elmer, Boston, MA, USA) according to Huang et al. (1985). Samples in study VIII were pre-digested according to Kumpulainen and Paakki (1987). The percentage of six major protein components of true proteins was also estimated in study VII on the basis of the amino acid data in Table 1 using overdefined linear regression and the sum of the least square method (Harville, 1997; Lay, 2006; Krutchkoff, 1970). The advantage of this method is that it is insensitive to the state of the proteins.

2.7.2 Functional analysis

Degree of denaturation (II)

The degree of denaturation (DD) was calculated as the ratio of the soluble whey protein at pH 4.6 and 6.7 according to Morr et al. (1985). Soluble whey protein was determined according to Lowry et al. (1951).

Heat stability (II)

Heat stability was measured according to McSweeney et al. (2004). The heat coagulation time (HCT) was the time of the onset of coagulation at 140°C, as described by Davies and White (1966).

Viscosity of DWP and model infant formula (II)
Viscosity of 1.5 g protein/100 g solutions of DWP and model infant formula (casein to whey protein ratio of 40:60) was determined as described in study II.

Water-holding capacity (II)
Water-holding capacity (WHC) was defined as the maximum amount of water bound by a unit of protein. The WHC analysis was carried out as described by Quinn and Paton (1979), modified by Rantamäki et al. (2000).

Emulsifying capacity (II)
Emulsifying capacity (EC) was defined by Harper (1991) as the maximum amount of oil that can be added to protein solution before the emulsion breaks. The EC was analysed according to Vuillemard et al. (1990). The analysis is reliable only in very dilute (<0.1%) protein solutions (Langley et al. 1988).

Emulsion stability (II)
The emulsion samples were centrifuged. The Emulsion stability (ES) was defined as the percentage of remaining emulsion after removing the supernatant according to Fligner et al. (1991) and Haque and Mozaffar (1992).

WPI gel hardness measurement (VIII)
CMP, WPI and α-LA fractions were produced with a chromatographic process (Outinen et al., 1995; Outinen et al., 1996). The protein fractions (Table 1, study VIII) were dialysed against distilled water and freeze-dried. The Na^+, Ca^{2+} and protein contents were taken into account when adjusting the mineral and protein concentrations of the solutions.

Based on the studies of Matsudomi et al. (1991), Schmidt et al. (1978), Schmidt et al. (1979), Mulvihill and Kinsella (1988), Zirbel and Kinsella (1988), Aquilera (1995) and Doi (1993), the experiments were carried out at pH 8.0, in Na^+- and Ca^{2+} -concentrations of 30-600 and 11-20 mM, respectively. The preparation and analysis of the gels is described in detail in study VIII.

2.8 Statistical analysis

One way analysis of variance (ANOVA) and the statistical significance of the results from each trial was carried out using Tukey HSD test with significance at $p<0.05$ using Statistica 7.1 (StatSoft. Inc., Tulsa, USA) software. Before the Tukey test, the material was tested for normality and homogeinity of variance with Shapiro-Wilk and Levene tests, respectively.

3 Results and discussion

3.1 Effect of HH, UF, UFHH and polymeric MF treatments on the recovery yields from cheese milk to whey (I, II)

The analysis of whey in study I was carried out with non-clarified whey to see the effect of the pre-treatments on residual fat or casein content. The samples in study II were obtained after clarification of whey. There was no significant difference in the recovery yield (RY) of casein from milk to whey, but the fat content of UF whey was significantly reduced, indicating that the elevation of protein content of milk by UF enhanched the transfer of fat from milk to cheese. Similar conclusion has been presented by Guinee et al. (1994).

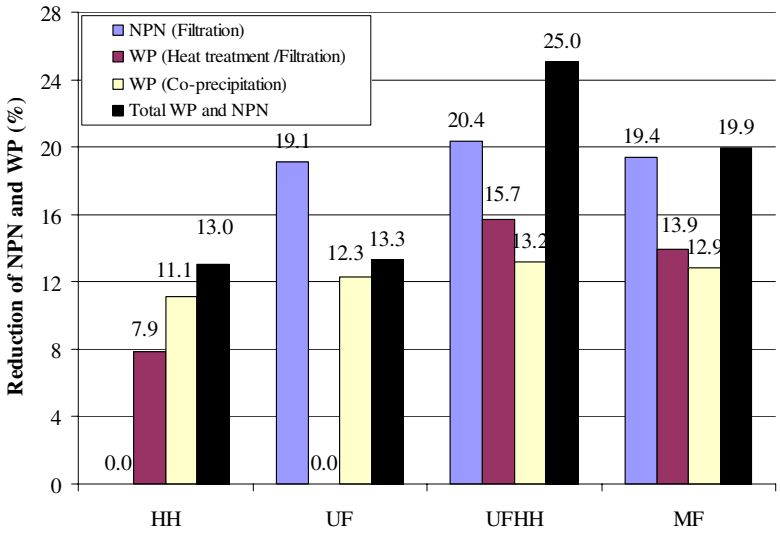


Figure 9. The effect of pre-treatment methods on the loss of non-protein nitrogen (NPN) and native whey protein (WP) during processing, expressed as percentage of original WP and NPN in milk. HH=partial high-temperature heat treatment; UF=Elevation of vat milk protein by ultrafiltration; UFHH whey=Elevation of vat milk protein by UF combined with high-temperature heat treatment. MF=elevation of vat milk protein by MF (CF=4, n=4). Filtration=Reduction of component in whey to UF or MF permeate. Heat treatment= Reduction in the quantity of native WP in vat milk after heat treatment. Co-precipitation=Reduction in the quantity of native WP after cheese production. Loss of NPN is calculated as protein equivalent (NPN×6.38).

According to the NPN and native whey protein (WP) mass balances (Figure 9), ca. 20% of NPN was lost into the MF and UF permeates. The co-precipitation of proteins to cheese was slightly enhanced by UF, but the net non-casein protein loss of 13.3% was comparable to the HH treatment (13.0%). With MF, in addition to NPN, 13.9% of WP was lost in the MF permeate, resulting in total loss of non-casein protein of 19.9%. Ultrafiltration induced denaturation of whey proteins only when combined with HH treatment, resulting in the highest loss of non-casein protein of 25.0%.

3.2 Effect of HH, UF, UFHH and polymeric MF treatment on the whey composition (I, II)

Water, lactose, minerals and NPN were partially removed from vat milk to UF permeate. WP was completely retained in the UF retentate and thus in the cheese milk. UF and MF processes produced 22% less whey compared to REF and HH. As the loss of protein from UF cheese milk to whey was only 13.3% (Figure 9), the protein concentration of UF whey was significantly elevated to 15.3% of TS (Table 2). Though the volume reduction of whey was equal in the MF process, the protein content of MF whey increased only to 14.3% of TS, due to loss of WP to the MF permeate. The HH treatment of the UF milk reduced the protein content of the UFHH whey to 14.2%, due to enhanced precipitation of the whey proteins in the cheese matrix.

Only UF milk without heat treatment produced whey with significantly different protein content of total solids. The NPN concentration was reduced because of permeation to UF permeate, whereas TP, WP, α-La and β-LG were significantly elevated because of the content of proteins in the UF retentate. However, the differences in relation to TP (Table 3), even with UF whey, were limited. The CMP content in whey was increased with all pre-treatments, indicating that the enhanced formation of κ-casein-β-LG –complexes did not significantly reduce the action of chymosin on κ-casein. The quantity of GMP in CMP was 57-65%, being in accordance with the previously published data (Casal et al., 2005; Vreeman et al., 1986; Lieske and Konrad, 1996). The amount of CMP formed ranged from 5.4 to 6.5 g/kg casein in studies I and II.

Table 2. Composition of various types of wheys, mean±standard deviation. REF whey=pasteurisation of milk (n=17); HH whey=partial high temperature heat treatment of milk (n=14); UF whey: elevation of vat milk protein by ultrafiltration (n=9); UF HH whey=elevation of vat milk protein by UF combined with high temperature heat treatment (n=17). MF whey=elevation of vat milk protein by MF (CF=4, n=4) TS=Total solids in whey, TP=Total proteins in whey, NPN=Non-protein nitrogen, α-LA=α-lactalbumin, β-LG=β-lactoglobulin. NPN-P=Non-protein nitrogen protein equivalent (N×6.38), CMP=total caseinomacropeptides, GMP=glycosylated caseinomacropeptides.

	REF whey	HH whey	UF whey	UFHH whey	MF whey*
TS (g/100g of whey)	5.2±0.2^{a}	5.1±0.2^{a}	5.2±0.2^{a}	5.1±0.2^{a}	5.2±0.1^{a}
TP (g/100g of whey)	0.70±0.03^{a}	0.65±0.04^{b}	0.79±0.04^{c}	0.73±0.03^{a}	0.75±0.03^{a}
Casein (g/100g of whey)	0.04±0.01^{a}	0.05±0.01^{a}	0.05±0.01^{a}	0.05±0.01^{a}	0.09±0.01^{b}
WP (g/100g of whey)	0.48±0.02^{a}	0.41±0.02^{b}	0.57±0.01^{c}	0.48±0.03^{a}	0.45±0.05^{a}
NPN (g/100g of whey)	0.30±0.01^{a}	0.30±0.04^{a}	0.30±0.01^{a}	0.30±0.01^{a}	0.31±0.01^{a}
α-LA (g/100g of whey)	0.09±0.01^{a}	0.08±0.00^{b}	0.10±0.01^{c}	0.09±0.01^{a}	0.09±0.01^{a}
β-LG (g/100g of whey)	0.26±0.02^{a}	0.23±0.03^{b}	0.31±0.01^{c}	0.25±0.04^{a}	0.23±0.06^{a}
CMP (g/100g of whey)	0.16±0.01^{a}	0.19±0.01^{b}	0.22±0.01^{c}	0.20±0.01^{c}	0.20±0.01^{c}
GMP (g/100g of whey)	0.09±0.01^{a}	0.12±0.01^{b}	0.14±0.01^{c}	0.13±0.01^{c}	0.12±0.01^{b}
TP (g/100g of TS)	13.4±0.1^{a}	12.7±0.1^{b}	15.2±0.1^{c}	14.2±0.2^{d}	14.3±0.2^{d}
Casein (g/100g of TP)	5.0±1.0^{a}	8.0±4.2^{a}	5.3±1.5^{a}	7.4±0.8^{a}	13.2±0.8^{b}
WP (g/100g of TP)	67.9±0.7^{a}	63.0±4.6^{b}	76.3±0.9^{c}	73.5±0.5^{c}	60.5±0.5^{b}
NPN-P (g/100g TP)	27.1±1.1^{a}	29.0±0.4^{b}	23.7±0.9^{c}	26.5±0.5^{a}	27.2±0.5^{a}
α-LA (g/100g TP)	12.5±0.7^{a}	12.3±0.3^{a}	12.8±0.5^{a}	12.4±0.5^{a}	12.4±0.5^{a}
β-LG (g/100g TP)	37.9±2.1^{a}	33.1±0.9^{b}	39.7±0.6^{a}	35.7±0.5^{a}	35.7±0.5^{a}
CMP (g/100g TP)	22.2±1.8^{a}	28.1±1.9^{b}	26.7±1.6^{b}	28.8±1.0^{b}	26.7±1.0^{b}
GMP (g/100g TP)	12.9±0.9^{a}	17.7±1.2^{b}	17.5±1.3^{b}	18.4±0.9^{b}	16.0±0.9^{b}
Ash (g/100g of whey)	0.44±0.03^{a}	0.43±0.02^{a}	0.45±0.01^{a}	0.44±0.02^{a}	n.a.
Na (mg/kg of whey)	320±10^{a}	312±20^{a}	310±20^{a}	310±20^{a}	n.a.
K (mg/kg of whey)	1310±60^{a}	1270±80^{a}	1300±100^{a}	1270±70^{a}	n.a.
Ca (mg/kg of whey)	330±20^{a}	350±20^{b}	350±20^{b}	360±20^{b}	n.a.
Mg (mg/kg of whey)	70±2^{a}	70±2^{a}	70±4^{a}	70±4^{a}	n.a.
Cl (mg/kg of whey)	900±50^{a}	870±50^{a}	880±60^{a}	880±30^{a}	n.a.
P (mg/kg of whey)	330±10^{a}	340±20^{a}	330±20^{a}	340±10^{a}	n.a.

Results within a row sharing the same superscript are not statistically different ($p<0.05$). *Unclarified whey (study I)

3.3 Effect of HH, UF and UFHH treatments of milk on the amino acid composition of WPC (I) and DWP (II) powders

The effects of HH, UF and UFHH treatments on the amino acid composition of WPC (study I) and DWP (study II) were studied. As was expected on the basis of the limited differences in the protein composition, the differences in the amino acid composition were limited.

Table 3. Composition of essential amino acids (g/100g of total amino acids) of WPC powders (I), mean±standard deviation, n=2. Wheys were obtained from renneted Edam vat milks, which were composed of cream mixed with standard, untreated milk, (REF whey), partially (25%) high heat treated milk and skim milk (75%) (HH whey), MF retentate and skim milk (MF whey) or UF retentate and skim milk (UF whey). Wheys were concentrated by 10 kDa UF and freeze-dried.

Amino acid	REF WPC	HH WPC	UF WPC
Thr	6.82±0.01[a]	7.02±0.16[a]	7.07±0.11[a]
Cys	2.46±0.13[a]	2.51±0.01[a]	2.47±0.04[a]
Val	5.91±0.16[a]	5.82±0.09[a]	5.65±0.16[a]
Ile	6.25±0.04[a]	6.32±0.01[a]	6.12±0.14[a]
Leu	10.32±0.07[a]	10.15±0.02[a]	10.11±0.04[a]
Met	2.03±0.06[a]	1.98±0.05[a]	1.96±0.01[a]
Tyr	2.72±0.06[a]	2.62±0.15[a]	2.65±0.05[a]
Phe	2.98±0.03[a]	2.97±0.08[a]	2.92±0.01[a]
His	1.77±0.01[a]	1.74±0.04[a]	1.73±0.02[a]
Lys	9.15±0.04[a]	8.98±0.06[a]	9.06±0.02[a]
Arg	2.29±0.03[a]	2.19±0.07[a]	2.21±0.04[a]
Trp	1.76±0.03[a]	1.72±0.01[a]	1.70±0.01[a]

Samples within a row sharing the same superscript are not statistically different ($p > 0.05$)

In study II, the clarified wheys were processed into demineralised whey powders (DWP). There were no significant differences in TP, “casein” (in practice mostly pH 4.6 insoluble whey proteins), mineral or fat contents of the DWPs. The only anomaly in the compositions of DWP compared to whey composition (study II) was the CMP content, which was 23 to 26 g/100 g of TS in whey, but only 19 to 22 g/100 g TS in DWP. Also the ratio of GMP to CMP was reduced in all DWPs compared to wheys, indicating a possibility that selective loss of GMP occurred during demineralisation. Since the heat load of DWP was extensive, the presence of heat-sensitive component in the GMP fraction was also considered. However, this possibility was ruled out based on the results obtained from heating (90°C for 7 minutes) experiment with whey: the heat treatment

did not affect the CMP or GMP results (data not shown). None of the pre-treatments affected the nutritional quality of the DWP powder: the differences in the amino acid composition were limited (Table 4).

Table 4. The amino acid composition of DWP in relation total amino acid content (g/100 g of amino acids), mean ± standard deviation (n=2). REF DWP=pasteurisation of milk, no high-temperature heat treatment, no UF; HH DWP=partial high-temperature heat treatment of milk; UF DWP=elevation of vat milk protein by UF; UFHH DWP= elevation of vat milk protein by UF combined with high-temperature heat treatment.

Amino acid	REF DWP	HH DWP	UF DWP	UFHH DWP
Thr	6.83±0.04[a]	7.01±0.10[b]	6.83±0.02[a]	7.13±0.03[b]
Cys	2.62±0.01[a]	2.52±0.00[a]	2.58±0.03[a]	2.49±0.11[a]
Val	6.20±0.01[a]	5.93±0.02[b]	5.96±0.02[b]	5.85±0.08[b]
Ile	6.37±0.01[a]	6.37±0.03[a]	6.35±0.00[a]	6.35±0.06[a]
Leu	10.11±0.03[a]	10.14±0.06[a]	10.21±0.03[a]	10.08±0.06[a]
Met	2.00±0.01[a]	1.96±0.01[a]	1.99±0.01[a]	1.95±0.04[a]
Tyr	2.33±0.01[a]	2.16±0.06[a]	2.22±0.01[a]	2.24±0.05[a]
Phe	2.66±0.01[a]	2.89±0.07[b]	2.90±0.02[b]	2.87±0.02[b]
His	2.12±0.02[a]	1.72±0.02[b]	1.75±0.02[b]	1.74±0.03[b]
Lys	8.95±0.01[a]	9.17±0.08[b]	9.21±0.04[b]	9.15±0.02[a]
Arg	2.25±0.01[a]	2.20±0.01[a]	2.30±0.01[a]	2.20±0.00[a]
Trp	1.79±0.02[a]	1.68±0.04[a]	1.71±0.03[a]	1.67±0.03[a]

Samples within a row sharing the same superscript are not statistically different ($p>0.05$)

As the CMP content of TP was elevated in all samples, the content of Tyr, Phe, His, Arg, Trp and Cys was expected to be decreased and the content of Thr increased. The results were contradictory. The Thr content was significantly elevated in HH and UFHH DWPs, but not in UF DWP. The content of His was significantly reduced in HH and UFHH DWP, as was expected. However, no logical explanation could be found to the low value of His in UF DWP. There was no statistically significant difference in the contents of Tyr, Cys, Arg and Trp, and the concentration of Phe was actually increased with all pre-treatment methods compared to REF DWP.

3.4 Effect of HH, UF and UFHH treatments of milk on the amino acid composition of ready-to-feed infant formula (II)

Compositional data of the HH, UF and UFHH DWP was used to calculate a model infant formula. The formula consisted of DWP, skimmed milk powder, lactose and cream to produce a standard ready-to-feed infant formula, with protein content of 1.5 g/100 g of formula, and with WP to casein ratio of 60:40. The result was compared to minimum allowed content of each essential amino acid required for infant formula by EU (Anon., 2006a). As expected, the contents of all essential amino acids of HH, UF and UFHH infant formulas were sufficient (Figure 10)

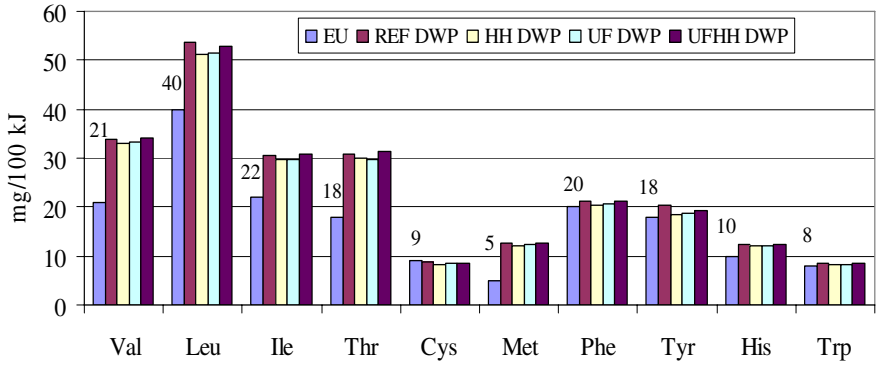


Figure 10. Effect of demineralised whey powder (DWP) produced from HH, UF and UFHH treatments of milk on the amino acid composition of ready-to-feed infant formula. REF=pasteurisation of milk, no high-temperature heat treatment, no UF; HH =partial high-temperature heat treatment of milk; UF =elevation of vat milk protein by UF; UFHH=elevation of vat milk protein by UF combined with high-temperature heat treatment. EU= Minimum content of essential and conditionally essential amino acids in infant formulae according to EU Commission directive 2006/141/EU (Anon., 2006).

3.5 Effect of the HH, UF and UFHH treatments on the functionality of the demineralised whey powders (II)

Degree of denaturation

There was no statistical difference in the degree of denaturation (DD) of the whey proteins in whey or in DWPs, though the mean figures were slightly elevated for ultrafiltered DWPs. The DD of proteins were considerably higher in all powders compared to DD of proteins in whey (Figure 11). The elevated degree of denaturation of DWP was explained by the severe pasteurisation of the whey concentrates before evaporation. The effect of partial heat treatment of cheese milk concerning the DD of the proteins of the DWP powder could not be detected.

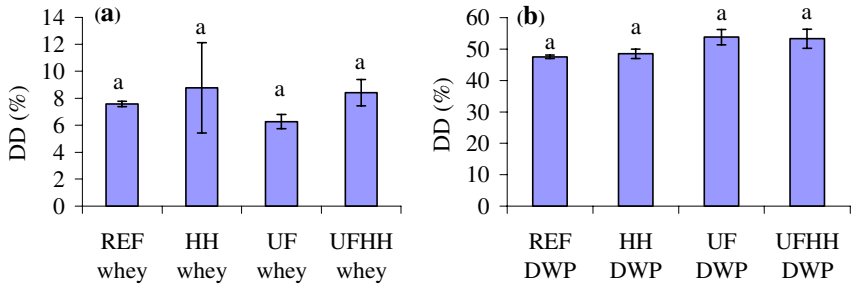


Figure 11. The degree of denaturation (DD) of whey proteins in whey (a) and demineralised whey powders (b) manufactured from whey produced from pasteurised cheese milk (REF), high-temperature heat treated milk (HH DWP), milk with elevated protein concentration (UF DWP) and high-temperature heat treated ultrafiltered milk (UFHH DWP), mean±standard deviation (n=6). Results with the same letter are not significantly different ($p>0.05$).

The degree of denaturation correlated well with the results of Law et al. (1994), who reported a DD of 40% for total whey proteins at comparable conditions of 80ºC for 5 min. Since ca. 25% of total whey proteins are soluble peptides (proteose-peptones and glycomacropeptides) at pH 4.6, maximum DD of total whey proteins is ca. 75% (de Wit 1989; Law et al. 1994). Hence, the DD of 40% of total whey proteins obtained by Law et al. (1994) equals DD of true whey proteins of ca. 53%. Moon and Mangino (2004) reported DD of 43-44% (at pH 4.6) after heat treatment of WPC at 84ºC for 60 s.

Heat stability

Ultrafiltration with or without high-temperature heat treatment improved the heat stability of DWP within the whole pH range (Figure 12).

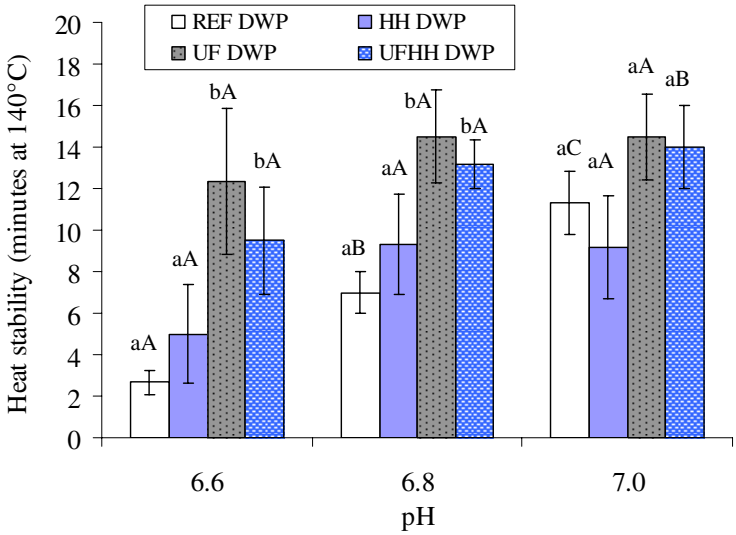


Figure 12. Heat stability of demineralised whey powders manufactured from whey produced from pasteurised cheese milk (REF), high-temperature heat treated milk (HH DWP), milk with elevated protein content (UF DWP) and high-temperature heat treated ultrafiltered milk (UFHH DWP), mean±standard deviation (n=6). Analysis results at given pH with the same small letter are not significantly different ($p>0.05$). Capital letters have been used when comparing the samples in different pH values, results with the same capital letter are not significantly different.

Improved heat stability may be due to elevated content of soluble protein aggregates formed by combined heat load of heat treatment of milk and evaporation and drying of DWP, indicated by the differences in the content of β-LG in whey and subsequent DWP (Tables 2 and 4), as well as by the marginally elevated degree of denaturation of UF and UFHH DWPs (Figure 11b). The effect of pH was less pronounced than expected: stability of all powders was lower at pH 6.6 than at the more stable area of pH 6.8 to 7.0 defined by McSweeney et al. (2004), but the difference was statistically significant only for REF DWP.

Viscosity

The viscosity of all solutions was very low, in a range of 1 cP and 2 cP at 60ºC and 25ºC, respectively. Pre-treatment of cheese milk appeared to have no significant effect on the viscosity of the DWPs, nor pre-heating in the oil bath. The average viscosity of the model infant formulas ranged from 1 to 1.5 cP at 60ºC, and ca. 2 cP at 25ºC. Again, the pre-treatment method of cheese milk or pre-heating had no significant effect on the viscosity of the DWPs. The viscosity range of all samples was acceptable for drinkable products, for which 20 cP is considered as the maximum acceptable viscosity (Moon and Mangino, 2004).

Water-holding capacity

Different pre-treatments did not have measurable effect on the water-holding capacity (WHC). Since there was no statistically significant differences in solubility, and as the increase of WHC has been observed to be strongly related to unfolding of the protein structure and subsequent increase in the degree of denaturation (Mangino, 1984), the result was as expected. WHC for all samples was 1.9 g H_2O /g protein, which was comparable with the results obtained by Heino et al. (2007), who reported WHC of ca. 1.7 g H_2O/g protein for a commercial spray dried WPC.

Emulsifying capacity

The maximum emulsifying capacity (EC) of REF, HH, UF and UFHH DWP was 0.96, 0.9, 0.65 and 1.00 g oil/mg protein, respectively, which was obtained by 0.025 or 0.05% protein solutions. This is in accordance with previous findings by Langley et al. (1988), who reported EC values of 0.05-1.0 mL oil/mg protein for WPI solution in similar conditions. The EC of medium heat skimmed milk powder (SMP MH) was 0.56 g oil/mg protein with a 0.05% protein solution, indicating that the EC of whey proteins was comparable to the EC of casein.

Emulsion stability

The emulsion stability (ES) of UF DWPs was slightly increased at elevated protein contents, though there were no statistically significant differences. Leman et al. (1988) found that the ES was increased with increased protein, and especially β-LG content. On the other hand, Yamauchi et al. (1980) found that the ES decreased with increasing ionic strength, especially at elevated concentrations of Na^+ and Ca^{2+}. The effect of elevated protein content may thus have been diminished by increased ionic strength of the

solution: the increase of the protein content also resulted in elevated ash content of the solution. The ES of the REF DWP was slightly reduced, possibly due to the elevated Na^+ concentration. However, the differences were negligible. The ES of all samples was 26.7-31.0%. The ES of the DWPs was comparable to SMP.

3.6 Effect of CF of ceramic microfiltration of cheese milk on the recovery yield of whey components and composition of whey (III, IV)

3.6.1 Permeability of the milk components

Casein was retained almost completely in the MF retentate at all CF values, resulting in an increase of casein to total solids (TS) from 78% (skim milk) to 92% (CF 10.5). Lactose was removed efficiently by diafiltration (DF) with water, increasing the total protein (TP) content of the retentate to ca. 80% of TS. β-LG was significantly more retained in the MF retentate than α-LA at all CF values, being in accordance with Tolkach and Kulozik (2004). However, Zulewska et al. (2009) found no difference in the retention of α-LA and β-LG with ceramic MF. At CF 1.4, the retention of WP was significant, indicated by the low protein content in the MF permeate (Figure 13). In addition to residual α-LA and β–LG, other whey proteins were also present in the MF retentate, as the recovery yield of total whey proteins (WP) from milk to whey was significantly elevated compared to the combined recovery yieds of α-LA and β-LG.

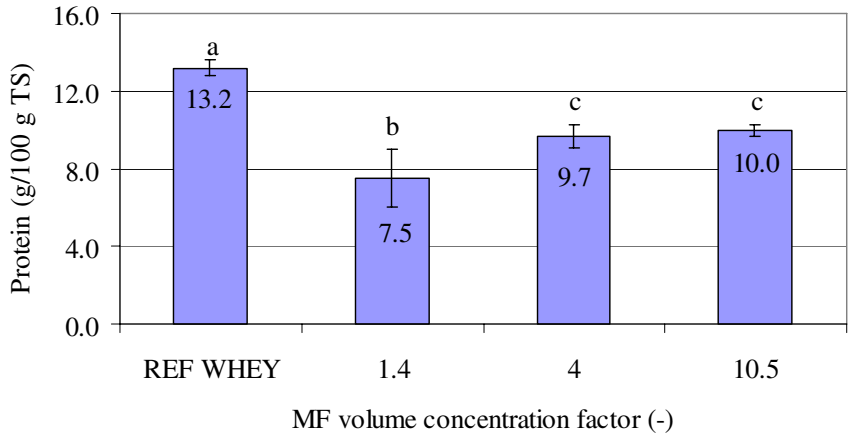


Figure 13. Protein content (g/100 g total solids) of the ceramic microfiltration (MF) permeates as a function of volume concentration factor (CF), mean±standard deviation (n=3). REF WHEY=reference whey. Results sharing the same superscript are not significantly different ($p>0.05$).

The 3.2-3.5%-unit difference between reference whey and DF permeate equals the amount of CMP present in the reference whey. The CMP content of the whey proteins was therefore approximately 23% of whey proteins, which is supported by Thomä-Worringer et al. (2006). As the CF was elevated to 10.5, the RY of TP from milk to whey decreased from 21 to 12%, as expected. However, statistically significant differences in the recovery yield of individual protein components could not be detected, due to the limitations set by the accuracy of analysis and dilute solutions.

3.6.2 Whey composition

Even after diafiltration (DF) to CF 10.5, there was a considerable amount of β-LG and other whey proteins in the whey (Figure 14). The CMP content in TP was elevated from 30% (CF 4) to 40% (CF 10.5). The average CMP content was 1.4 g/L of whey, which was in accordance with the literature data (Swaisgood, 2003a; Kreuss and Kulozik, 2006). The total yield of CMP varied from 43 to 55 g/kg casein.

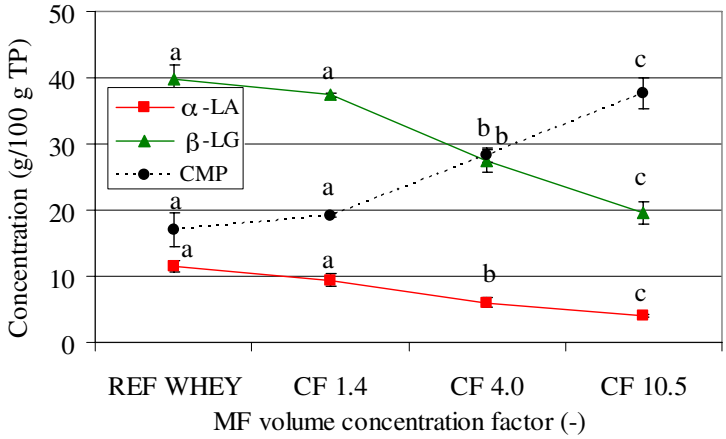


Figure 14. Ceramic microfiltration (MF). Protein composition as a function of volume concentration factor CF (-), mean±standard deviation (n=3). CF 1.0=reference whey. α-LA=α-lactalbumin, β-LG=β-lactoglobulin, CMP=caseinomacropeptides. Results sharing the same superscript are not significantly different ($p>0.05$).

3.7 Effect of diafiltration of milk with polymeric MF membranes on the recovery yield of whey components and whey composition (V-VII)

3.7.1 Permeability of whey proteins (V)

The protein composition of the MF permeate varied according to the CF (Figure 15). The content of whey proteins obtained maximum value at CF 4. This was to be expected since the protein content reached the maximum also in the retentate. During diafiltration, the injection of the UF permeate was equal to the MF permeate formation stabilising the casein content in the retentate. Immediately at the beginning of the DF with UF permeate, the content of whey proteins in the MF permeate began to decrease, reaching negligible levels after CF 70. The mass flux of the whey proteins after CF 70 was considered not useful for practical purposes. The residual α-LA and β-LG were retained in the retentate probably due to the formation of concentration polarisation layer near membrane surface. Therefore, additional DF would have resulted in limited decrease in whey protein content in the retentate.

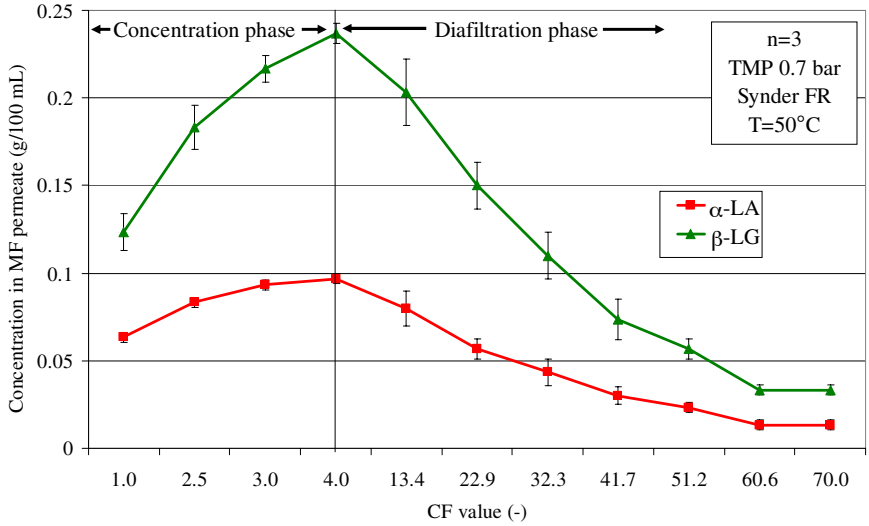


Figure 15. The α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) content in the permeate of polymeric microfiltration (MF) permeate as a function of volume concentrationt factor (CF).

The UF permeate, which was used as a DF medium, contained lactose, NPN and minerals approximately in the amounts found in milk. Consequently, the TS, lactose and NPN contents of the retentate and permeate were practically constant during the whole duration of filtration. The residual fat of skimmed milk was completely retained in the retentate. The permeate was clear, also indicating that permeation of fat and micellar casein was limited. The retention of casein was at least 98%; more accurate estimation was difficult due to the large quantity of permeate and the limits set by the accuracy of analysis. However, traces of casein were detected in the permeate.

The β-LG content in the MF retentate was reduced from 9.6 to 0.9% of TP (93.5% reduction). The α-LA content was reduced from 3.9 to 0.2% (95.8% reduction). There was no significant difference between the permeability rates of α-LA and β-LG. The permeability of α-LA and β-LG was considerably higher for this type of polymeric MF membrane than previously reported (Lawrence et al., 2008; Zulewska et al., 2009). The filtration at 50°C clearly enhanced the permeability and selectivity of the membrane. Govindasamy-Lucey et al. (2007) filtrated skimmed milk at <7°C using the Synder

FR2B membranes, also used in this study. Despite two DF steps, the permeation of proteins was only 36%, of which ca. 22% consisted of β-casein.

Most of α-LA and β-LG were transferred into the permeate already after the first diafiltration step (CF 10), further diafiltration did not significantly decrease their content in the retentate. The WP content of total proteins (TP) was reduced from 15.4% (skimmed milk) to 4.5% (CF 70 retentate), equalling only 77% reduction. This indicated that the permeation of large MW whey proteins was reduced, as was observed also with ceramic membranes (IV). The retention of high MW whey proteins has been observed with both ceramic (Jost et al., 1999) and polymeric MF membranes (Lawrence et al., 2008; Neocleus et al., 2002; Nelson and Barbano, 2005a; Zulewska et al., 2009).

3.7.2 Milk composition obtained by polymeric microfiltration (V)

The vat milks were composed of the MF retentates. Compared to the reference milk, the contents of WP, α-LA and β-LG in the CF 70 milk were reduced by 70, 87 and 88%, respectively, again indicating reduced permeation of large MW whey proteins in the 800 kDa MF permeate. The calcium content was significantly elevated (34 to 35 mg Ca/g protein) in all MF milks compared to reference (32 mg Ca/g protein), due to calcium bound to micellar casein.

3.7.3 Recovery yield of whey components from milk to whey (VI)

The RY of fat and Ca in whey was significantly decreased in all MF milks. However, the CF (the content of whey proteins) had no effect, supporting the findings of Brandsma and Rizvi (2001). The recovery of total protein was naturally enhanced by the increasing CF, as the ratio of casein to TP increased with increasing CF. The RY of β-LG of reference whey was significantly higher compared to all MF wheys, indicating that elevated casein content might increase the occurrence of β-casein-β-LG complex formation as described by Maubois (2002). There was no strong evidence of enhanced occlusion of native whey proteins into casein matrix, which is in accordance with the results obtained in study I.

The concentration factor or elevation of protein concentration had no significant effect on the concentration, yield or quality of CMP. The yield was 48 to 55 g/kg casein. The ratio of GMP in CMP ranged from 56% to 58%.

3.7.4 Whey composition (VI)

The volume and mass of the MF wheys were reduced by approximately 20% in comparison to reference whey, as was expected. The quantity of TS in MF wheys was significantly decreased, as lactose was partly removed into the MF permeate. Although the quantity of fat was significantly reduced in all MF wheys, the content in whey was comparable to REF whey, as the whey volume was reduced.

There was still 66% of TS, 47% of TP and 34% of WP in the CF 70 whey compared to the amount present in the reference whey (Figure 16). Though the casein to TP ratio increased in MF wheys, the quantity of residual casein in all MF wheys decreased compared to the reference whey (Figure 16). This indicates that elevation of casein content by MF could enhance the casein yield, as suggested also by Maubois (2002).

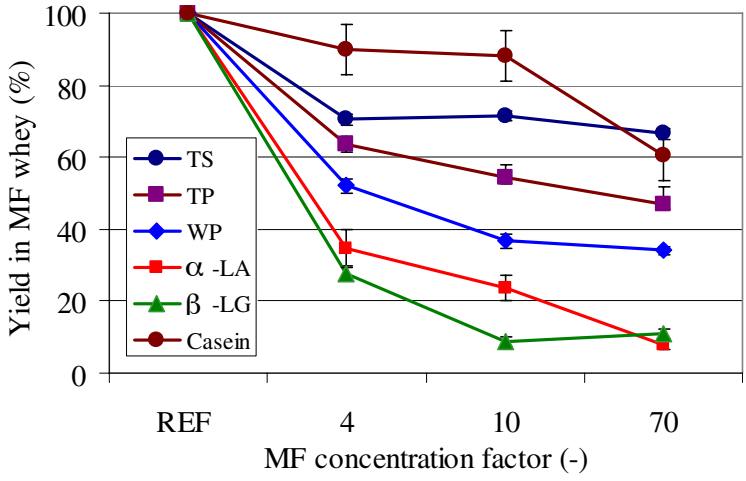


Figure 16. Yield of total solids (TS), total protein (TP), casein, whey proteins (WP), α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) in wheys obtained from milk produced from microfiltered (MF) milk retentate. REF=reference whey. The yield was calculated by comparing the mass of the component in MF whey to the mass of the component in the REF whey as a function of volume concentration factor (CF) of the MF, mean ± standard deviation (n=4).

Although NPN was lost to MF permeate, new NPN was introduced into the MF system with the DF medium, which was the UF permeate. Simultaneously, WP was removed

from the retentate. Hence, the NPN content in TP was increased significantly as CF increased until CF 10 (Figure 17). The WP to TP ratio was not significantly different between CF 4, CF 10 and CF 70 wheys.

The CMP to (α-LA+β-LG) ratio increased from 0.43 (REF whey) to 4.3 (CF 70 whey). However, the CMP to WP ratio was approximately 1 (Figure 17). Hence, it is evident that, other whey proteins were also present in the MF milk and released into whey after renneting, as suggested also by Jost et al. (1999).

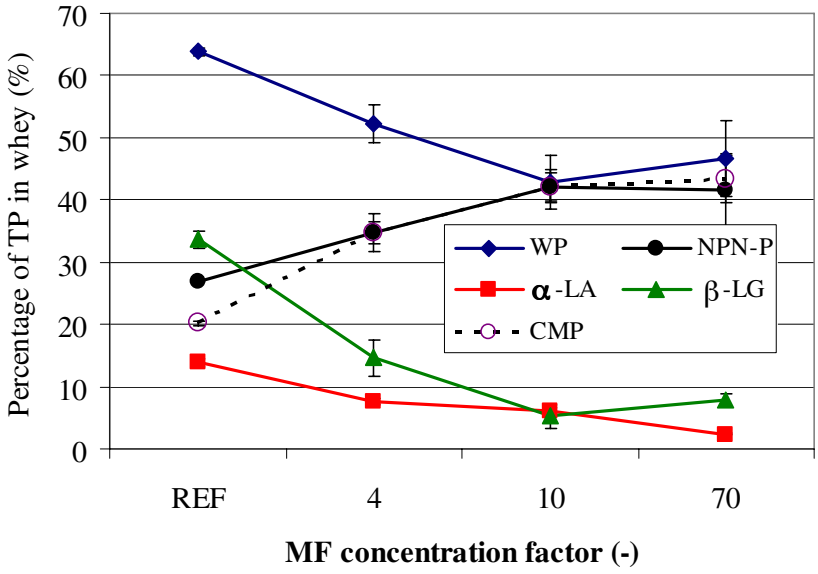


Figure 17. Protein composition in MF wheys as a function of volume concentration factor (CF), mean±standard deviation (n=4). The MF wheys were obtained from cheese milk produced from microfiltered (MF) milk retentate. REF=reference whey, TP=total protein, WP=whey proteins, NPN-P= (NPN×6.38), α-LA=α-lactalbumin and β-LG=β-lactoglobulin, CMP= caseinomacropeptides.

3.7.5 WPC composition (VII)

The casein content in the MF WPC powders was increased from 4% (REF WPC) to 6-7% of TP. This was due to the decreased amount of whey proteins. The content of α-LA

of TP was reduced from 15% to 4% (WPC CF 70); β-LG from 48% to 14%. The whey protein (WP) content of total protein was reduced only from 89% to 77%. The CMP content was elevated in all WPC powders produced with MF, as expected. With CF 10, the content of CMP was increased from the reference WPC level of 23% to 48% (of TP), and was further increased to 69% in WPC CF 70. This was also shown as increased NPN content of total protein from the reference level of 8% to 14% of total protein in WPC CF 70, as GMP belongs partially to the NPN fraction (van Bockel, 1993). According to the SDS-PAGE analysis of the WPC powders, large whey proteins were present in the MF WPC powders. Additionally, their relative content was significantly reduced in NWPC, LF was nearly absent. The effect of CF on the mineral content was limited.

Amino acid composition of the MF WPC powders

Since CMP and GMP lack amino acids Tyr, Phe, and Trp, and are rich in Thr and Ile (Table 1), significant differences between the REF and MF WPC powders were expected concerning these amino acids. The Thr content was indeed statistically higher and Trp content already lower at CF 4 (Table 5). Therefore, MF WPC would be less suitable for infant nutrition where decreased levels of Thr and elevated levels of Trp are currently preferred (De Wit, 1998). The abundance Phe, Tyr and Trp in the MF WPC powders suggested a presence of significant amount of residual whey proteins. The amino acid profile of NWPC was significantly different from the WPC powders. The absence of CMP in NWPC was indicated by the reduced concentration of Thr and Ile and the increased concentration of aromatic amino acids

Table 5. Essential amino acid composition (g/100 g of total amino acids) of whey protein concentrates (WPC), mean ± standard deviation (n=3). The whey was obtained from Edam cheese production, in which 800 kDa microfiltration (MF) retentates with different concentration factors (CF) was used. REF WPC=reference whey produced from cheese whey, NWPC= WPC produced from the 800 kDa MF permeate.

	REF WPC	WPC CF 4	WPC CF 10	WPC CF 70	NWPC
Thr	6.82±0.01^{a}	8.36±0.24^{ab}	9.11±0.98^{bc}	10.62±0.21^{c}	4.66±0.05^{d}
Cys	2.46±0.13^{a}	1.93±0.04^{ab}	1.61±0.38^{b}	1.21±0.10^{b}	3.21±0.09^{c}
Val	5.91±0.16^{a}	6.55±0.01^{ab}	6.90±0.39^{b}	7.00±0.06^{b}	4.77±0.15^{c}
Ile	6.25±0.04^{a}	6.76±0.03^{a}	7.05±0.48^{a}	7.26±0.24^{a}	5.73±0.09^{c}
Leu	10.32±0.07^{a}	8.75±0.22^{ab}	7.85±1.06^{bc}	6.52±0.06^{c}	11.94±0.09^{d}
Met	2.03±0.06^{a}	1.92±0.02^{a}	1.85±0.37^{a}	1.99±0.05^{a}	2.06±0.01^{a}
Tyr	2.72±0.06^{a}	2.29±0.09^{ab}	2.04±0.33^{ab}	1.58±0.08^{b}	3.38±0.02^{c}
Phe	2.98±0.03^{a}	2.75±0.06^{a}	2.67±0.16^{a}	2.29±0.01^{b}	3.38±0.02^{c}
His	1.77±0.01^{a}	1.79±0.05^{a}	1.73±0.09^{a}	1.40±0.10^{b}	1.97±0.03^{c}
Lys	9.15±0.04^{a}	8.35±0.10^{ab}	7.92±0.36^{b}	7.52±0.15^{c}	9.72±0.03^{d}
Arg	2.29±0.03^{a}	2.25±0.08^{a}	2.20±0.22^{a}	1.95±0.06^{a}	2.26±0.06^{a}
Trp	1.76±0.03^{a}	1.30±0.05^{b}	1.07±0.26^{b}	0.78±0.03^{b}	2.22±0.04^{c}

Samples in the same row sharing the same superscript are not statistically different ($p > 0.05$)

Calculated protein composition according to amino acid analysis data

The results obtained with linear regression analysis of the amino acid composition (Table 6) supported the results obtained by SDS-PAGE. The calculated relative concentration of IgG in MF whey indeed significantly increased with increasing CF. The BSA content in the WPC powders remained relatively constant, indicating that the permeability of BSA was considerable. This was supported also by the considerable amount of BSA in the NWPC powder. The calculated contents of α-LA, β-LG and CMP were also compared with results obtained by HPLC (Table 6). The content of α-LA correlated well with the HPLC analysis results. The calculated β-LG content was considerably higher, indicating that some denaturation occurred during filtration in the retentate, and the major part of "casein" is in fact denatured β-LG. As the proteins were completely in a native form in the MF permeate, the correlation of calculated and analysed β-LG in the NWPC was very good. The correlation of calculated and analysed CMP was reasonably good, although the difference in WPC CF70 was considerable. This may be due to difficulties in interpreting the multiple GMP peaks in the HPLC analysis. To support the results obtained from WPC powders REF to CF 70, the relative concentrations of BSA and IgG were considerably decreased in the NWPC powder.

Lactoferrin was not detected in the NWPC or WPC powders, possibly due to low concentration of the protein, or enhanced co-precipitation to cheese.

Table 6. Statistical estimate of the whey protein (WP) composition of the WPC powders based on the amino acid data, mean±standard deviation*. CF=concentration factor of the 800 kDa microfiltration (MF) retentate used in Edam cheese production. NWPC=WPC produced from the 800 kDa MF permeate. REF WPC=WPC produced from cheese whey. α-LA=α-lactalbumin, β-LG=β-lactoglobulin, BSA=bovine serum albumin, CMP=caseinomacropeptides, IgG_1=immunoglobulin G_1, LF=lactoferrin.

	REF	WPC CF 4	WPC CF 10	WPC CF 70	NWPC
α-LA	15±4	9±4	4±4	2±4	23±4
α-LA**	14±1	9±0	8±2	5±2	28±1
β-LG	53±1	44±6	33±6	27±6	69±7
β-LG**	41±6	20±2	11±9	5±2	68±4
CMP	20±3	30±3	44±3	49±3	4±3
CMP**	24±1	42±4	48±2	64±1	0
BSA	12±6	10±6	11±6	12±6	8±6
IgG_1	3±3	8±4	9±4	12±4	0±3
LF	0	0	0	0	0

* The standard deviation of the result is calculated on basis of the accuracy of the amino acid analysis method according to Krutchkoff (1970).** Mean±standard deviation (n=3) analysed by the HPLC method (Thomä et al., 2006).

3.7.6 Effect of the MF treatment of milk on the amino acid composition of infant formula (I, VII)

Compositional data of the MF WPC powders from studies I and VII were used to calculate a model infant formula as described in 3.4. As expected on basis of the WPC amino acid data, the quantities of Cys and Trp failed to comply with the EU standard (Anon., 2006a) already at CF 1.4-4. At CF 70 this was a case also with Tyr and Phe. The quantity of Leu also decreased close to the minimum allowed concentration at CF 70 (Figure 18). The amino acid profile of infant formula produced from NWPC was significantly better compared to formula produced with WPC powders originating from cheese whey.

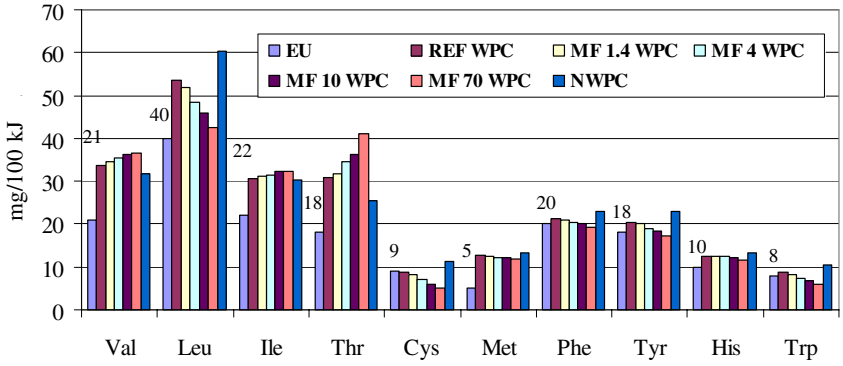


Figure 18. Whey protein concentrate (WPC) powder produced from whey from microfiltered (MF) milk. Effect of volume concentration factor (CF) on the amino acid composition of infant formula. REF=traditional processs, no MF treatment; MF 1.4-70=whey from cheese process in which MF retentate with CF 1.4-70 was used. EU= Minimum content of essential and conditionally essential amino acids in infant formulae according to EU Commission directive 2006/141/EU (Anon., 2006). NWPC=native WPC obtained from MF permeate.

3.8 Effect of CMP on the whey protein isolate gel hardness (VIII)

As the ionic environment of the gel have a strong impact on the gel characteristics, the effect of CMP content in the gels produced with whey protein isolate was studied in strictly controlled ionic environment. The Na^+ concentration of the protein gels was adjusted to 218 mM, the Ca^{2+} concentration varied from 11.1 to 19.6 mM. Regardless of the Ca^{2+} concentration, the WPI gel strength significantly decreased as the content of CMP increased (Figure 19). This is in accordance with Britten and Pouliot (1996), who observed significant decrease in the gel strength of cheese whey concentrate in comparison with NWPC in similar conditions (15 mM Ca^{2+}). The Ca^{2+} concentration had no significant effect on the gel hardness, though there was a slight increase in the mean hardness at 19.6 mM Ca^{2+}.

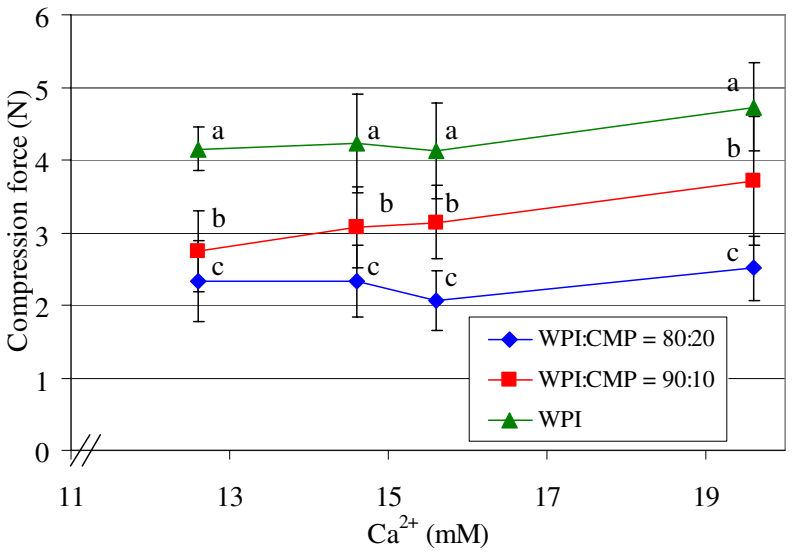


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Figure 19. Gel hardness of the caseinomacropeptide (CMP) enriched whey protein isolate (WPI) gels as a function of Ca^{2+} concentration in 218 mM Na^{+} solution, mean±standard deviation, n=8. Results sharing the same superscript are not statistically significantly different (p>0.05).

The decrease in the compression force could be thought to be caused solely by the decrease in the concentration of the functional protein β-LG, which was ca. 65% (WPI without CMP addition) to 59% (20% CMP addition). To verify this, the β-LG concentration was adjusted to 50% of total protein with α-LA and CMP ranging from 10 to 40%. As expected, the compression force was the lowest for the gel with 40% CMP. However, there was little difference in the hardness of the gels with 10 and 20% CMP. All gels were opaque-white, as expected in the prescence of 218 mM Na^{+}. No significant effect on texture was observed. Interaction of α-LA with β-LG was clearly stronger than interaction between CMP and β-LG, supporting the findings of Rojas et al. (1997). Even though the number of free thiol groups (i.e. same β-LG concentration) was constant, decrease in the content of α-LA significantly reduced the gel network formation properties. The effect of ionic environment was also significant: all CMP-enriched gels were much harder in all Ca^{2+} concentrations in the prescence of 218 mM Na^{+} than the WPI gels in the prescence of 30 mM Na^{+}.

4 Conclusions

High-temperature heat treatment (HH) of cheese milk had no significant effect on the content of fat, NPN or total proteins (TP) of HH milk. In HH whey, the quantity of native whey proteins (WP) and α-LA was reduced by approximately 10%, and β-LG by 20%, and the relative concentration of CMP and "casein" (denatured WP) was elevated. However, the HH treatment did not have significant effect on the nutritional value of the HH whey. The amino acid compositions of the WPC and DWP powders produced from HH whey were comparable to reference powders.

Ultrafiltration (UF) of vat milk produced 22-24% less whey by comparison to the reference processes (studies I and II). In the UF process, protein denaturation was negligible, but approximately 12.3% of native WP was enclosed in the cheese curd and thus removed from the whey fraction. Approximately 20% of NPN was lost in the UF permeate, totalling non-casein loss of 13.3%. As the recovery yield (RY) from cheese milk to whey was not increased in proportion to volume loss, the protein concentration, as well as the concentration of individual whey proteins α-LA, β-LG and CMP was significantly elevated. Recovery of whey proteins and fat to cheese was enhanced by UF. However, the loss of whey proteins into the cheese matrix did not have a significant effect on the nutritional value of the UF whey. In relation to the total amino acids, the amino acid composition of the WPC and DWP powders produced from UF whey were comparable to reference powders.

The combination of HH and UF (HHUF) of vat milk produced whey with significantly (25%) reduced mass and total solids, whereas the whey protein recovery from vat milk to whey was reduced by 20%, compared to refence processes. HH treatment of high-protein UF milk reduced the concentration of the protein and β-LG in UFHH whey close to the concentration of reference whey. The concentrations of α-LA and CMP, being more heat stable, remained elevated compared to reference whey. Despite the slight alteration in the protein profile, the amino acid composition of the DWP powders produced was comparable to the amino acid profile of the reference DWP. The ready-to-feed model infant formulas produced with HH, UF and UFHH DWPs were in accordance with the specifications set by EU legislation. The HH, UF and HHUF treatments had no negative effect on the functional characteristics of the DWP powders. HH treatment improved the heat stability of the DWP powders.

Elevation of casein concentration of cheese vat by microfiltration (MF) milk enhanced the recovery of casein and fat to cheese. The recovery was not affected by volume concentration factor. The content of α-LA and β-LG could be effectively reduced in the polymeric MF retentate using volume concentration factor (CF) of 70. However, large MW whey proteins were retained both in the ceramic and polymeric MF retentates, and could not be removed even with considerable diafiltration. Consequently, cheese whey with significantly altered protein and amino acid composition was produced. The nutritional quality of the MF whey, as well as the model ready-to-feed infant formulas produced threof, was impaired already with low CF values. Increase of CMP concentration in relation to α-LA and β-LG, as with MF whey, significantly reduced the whey protein gel hardness in all ionic environments.

HH and UFHH treatment of cheese milk produced whey of which acceptable DWP and WPC could be produced. UF treatment was also well suited for DWP production after standardisation of protein content. It appears that all whey protein components were lost approximately in the same proportion, resulting in comparable amino acid compositions. MF treatment had a negative effect on the composition as well as on the nutritional and fuctional characteristics of whey products. The nutritional quality and fuctional characteristics of the "native" MF permeate are excellent, but the utilisation of the MF cheese whey remains a challenge for the milk processing industry.

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